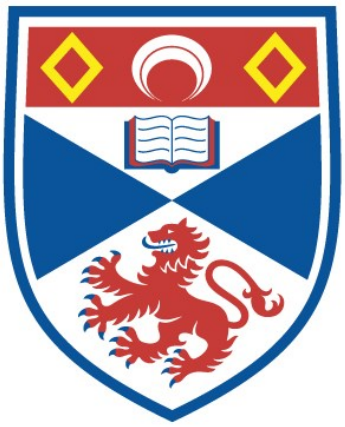
STUDIES ON THE MOLECULAR ORGANISATION OF CARTILAGE PROTEOGLYCANS

William Howard Stimson

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1970

STUDIES ON THE MOLECULAR ORGANISATION OF CARTILAGE PROTEOGLYCANS

being a thesis presented by

WILLIAM HOWARD STIMSON

to the University of St. Andrews
in application for the degree of Doctor of Philosophy

Department of Biochemistry.
University of St. Andrews.

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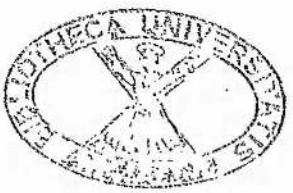

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D E C L A R A T I O N

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry in the United College of St. Salvator and St. Leonard, the University of St. Andrews, under the direction of Dr. A. Serafini-Fracassini.

C E R T I F I C A T E

I hereby certify that William Howard Stimson has spent nine terms engaged in research work under my direction, and that he has fulfilled the conditions of Ordinance No. 16 (St. Andrews) and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

ACADEMIC RECORD

I matriculated at the University of St. Andrews in October, 1964, and graduated with the degree of Bachelor of Science, First Class Honours in Biochemistry, in June, 1968. My subsidiary subjects were: Chemistry, Physics and Zoology at 1st B.Sc. level and Chemistry at 2nd B.Sc. level. In October 1968, I matriculated as a research student in the Department of Biochemistry, University of St. Andrews.

ACKNOWLEDGEMENTS

I should like to express my gratitude to Dr. A. Serafini-Fracassini for all his help and encouragement throughout this work.

I also wish to thank Mrs. L. Serafini-Fracassini for carrying out the Amino Acid Analyses, and Miss P. Keracher for typing.

C O N T E N T S

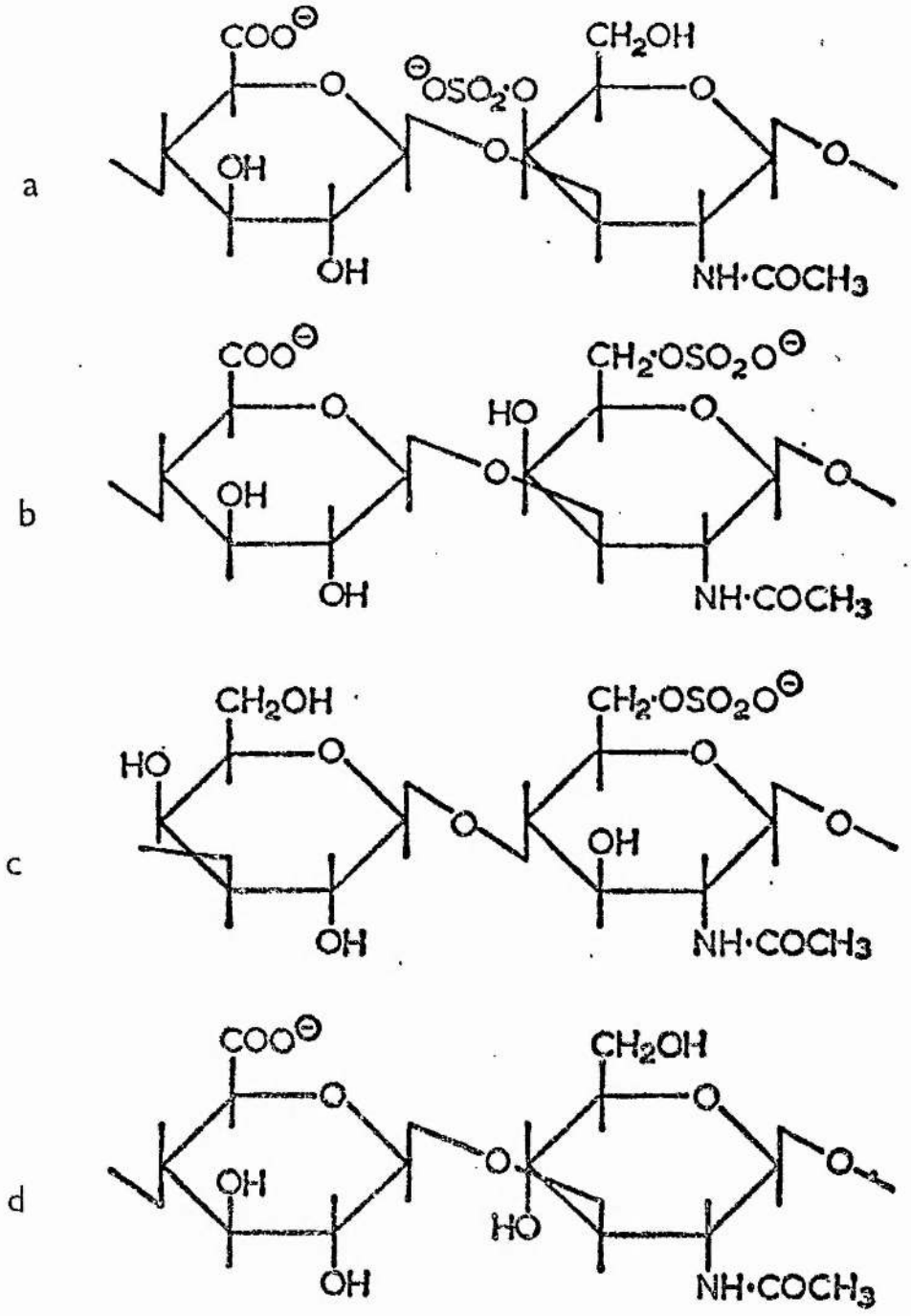
GENERAL INTRODUCTION

The two major components of the extracellular phase of hyaline cartilage (e.g. bovine nasal cartilage) are collagen and a complex of sulphated glycosaminoglycans and non-collagenous proteins called proteoglycans. The term proteoglycan refers to a macromolecule in which one or more polysaccharide chains are covalently bound to one or more protein chains, the carbohydrate moiety being the dominant feature of the macromolecule.

There are four cartilage glycosaminoglycans, chondroitin 4-sulphate, chondroitin 6-sulphate, keratan sulphate and hyaluronic acid. The structure of the repeating units of these components is shown in Fig. 1, and their chemistry is well documented. (Jeanloz 1963; Brimacombe and Webber, 1964; Muir, 1964; Bhavanandan and Meyer, 1966, 1967).

Protein-polysaccharide can readily be obtained from hyaline cartilage by high-speed homogenization in water (Malawista and Schubert, 1958). It can then be separated, by centrifugation (Gerber, Franklin and Schubert, 1960), into two fractions: a light fraction (proteoglycan) and

Figure 1



Structure of repeating units of cartilage glycosaminoglycans

(a) Chondroitin 4-sulphate
(b) Chondroitin 6-sulphate
(c) Keratan sulphate
(d) Hyaluronic acid

TABLE 1

ANALYSIS OF PROTEOGLYCAN ISOLATED FROM BOVINE NASAL CARTILAGE

(Luscombe and Phelps 1967a)

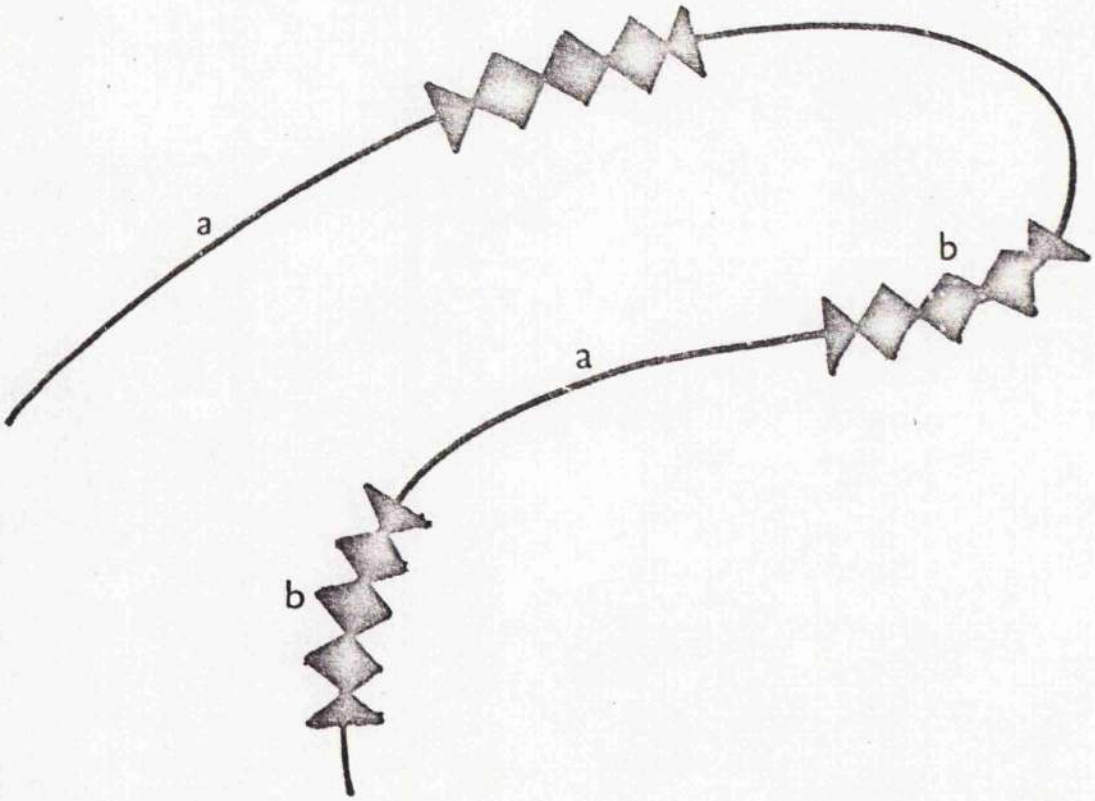
		% of dryweight
Hexuronic acid (as K salt)		30.0
N-acetylgalactosamine		23.7
N-acetylglucosamine		2.9
Protein	Biuret	19.8
	Folin-Phenol	14.7
Galactose		4.4
Fucose		0.25
Glucose		0.17
Mannose		0.36
Xylose		0.41
Sialic acid		0.14
Sulphur (as KSO_3)		16.6

a heavy fraction (collagen-proteoglycan complex, Schubert 1965). The proteoglycan bound to collagen in the heavy fraction could be identical to that contained in the light fraction, although this problem has not yet been resolved. Table 1 shows a typical analysis of proteoglycan (light fraction) purified by cetylpyridinium chloride precipitation from bovine nasal cartilage.

It is evident from the analytical data that chondroitin sulphate is the major glycosaminoglycan component although the glucosamine, neutral sugar and sialic acid contents show that both keratan sulphate and glycoproteins are present and these account for approximately 14% of the total carbohydrate.

In 1956, Webber and Bayley estimated the molecular weight of calcium chloride-extracted chondromucoprotein to be 1×10^6 by using sedimentation ultracentrafugation and viscometry techniques. The model they proposed for the complex was that of a random coil consisting of 20 chondroitin sulphate molecules, of molecular weight approximately 4×10^4, linked end to end by polypeptide chains (Fig. 2). The random coil conformation was confirmed by Bernardi (1957 a,b), but the molecular weight of his preparation was 1.98×10^6. However, Mathews and Lozaityte (1958)

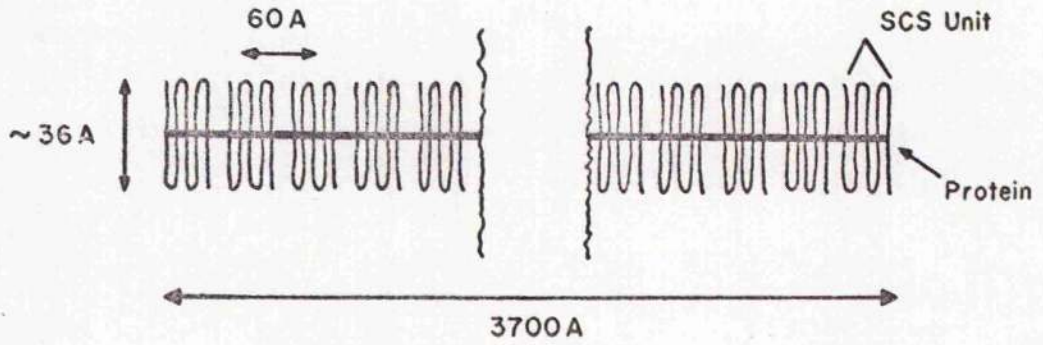
FIGURE 2



Model proposed by Webber and Bayley (1956)

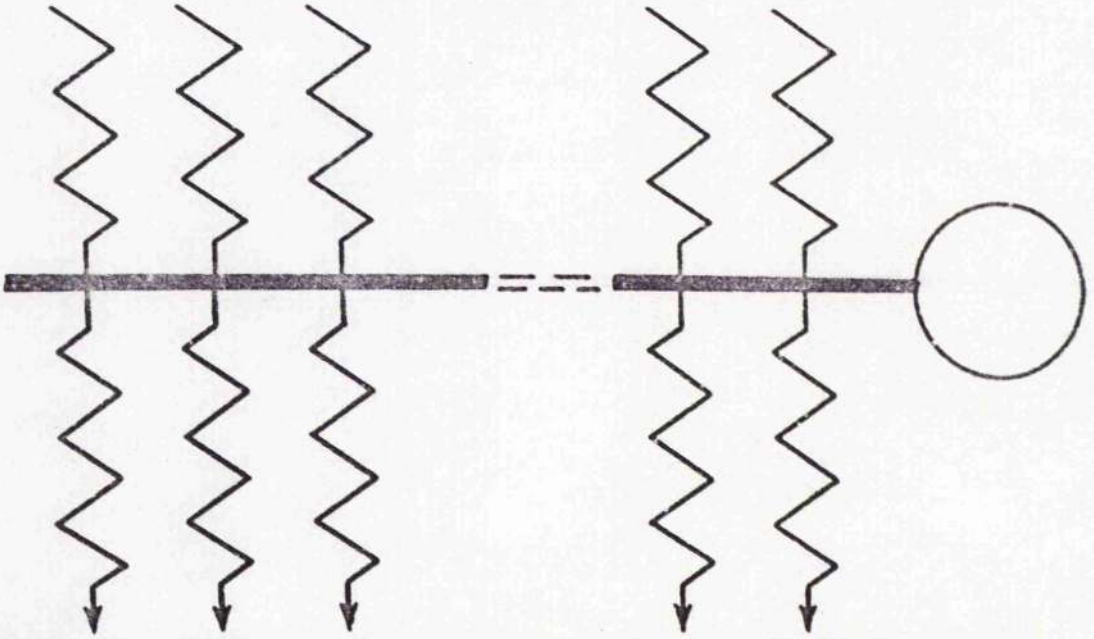
(a) protein (b) chondroitin sulphate

FIGURE 3



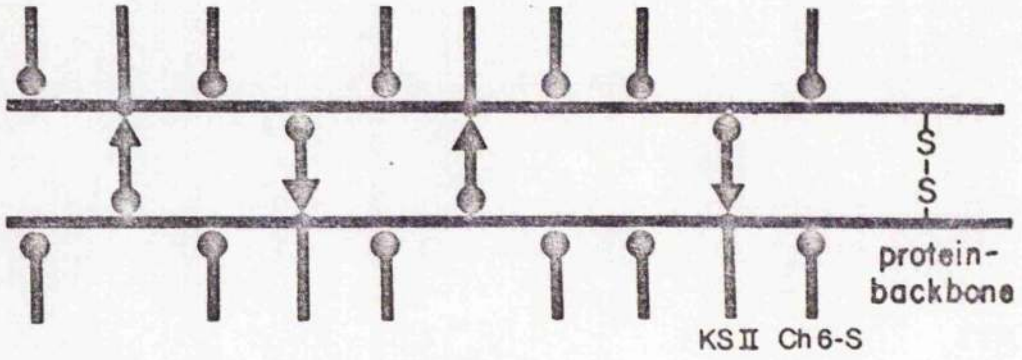
Model proposed by Mathews and Lozaityte (1958)

FIGURE 4



Model proposed by Partridge, Davis & Adair,(1961). The heavy line represents the protein core carrying at least 23 chondroitin sulphate chains. These are connected by a single linkage which does not involve the terminal reducing residue (indicated by arrowheads). The circle represents a second polysaccharide material which is rich in galactose and glucosamine.

FIGURE 5



Model proposed by Meyer (1970)

obtained data from light-scattering and viscometry indicating a rod-like macromolecule of molecular weight 4×10^6. The authors proposed a comb-like model in which the protein moiety was visualised as a core, 3 700 Å long, with 62 chondroitin sulphate chains uniformly distributed along it. (Fig. 3). Confirmation of this 'continuous protein core hypothesis' was given by Cessi and Bernardi (1965) through studying the kinetics of degradation of proteoglycan using hyaluronidase and papain. They found that hyaluronidase did not cause a large drop in the molecular weight of the proteoglycan, as did papain, thus disproving an 'end-to-end' model. Partridge, Davis and Adair (1961) suggested that the proteoglycan, in fact, consisted of smaller units of molecular weight 7.5×10^5 and that each unit was made up of a protein core, of molecular weight 120 000, carrying 23 chondroitin sulphate chains, each with a molecular weight of 28 000.(Fig. 4).

Serafini-Fracassini and Smith (1966) treated proteoglycan with bismuth nitrate in acetone and examined the precipitate by electron microscopy. They found beaded strands 1 100 Å to 1 500 Å long, composed of 20-25 particles with an average diameter of 30 Å. Each row was interpreted

TABLE 2

	Proteoglycan x 10^6	Protein Core x 10^4	Chondroitin Sulphate Chain x 10^4	Nos. of Polysacc. Chains	% Protein by wt.
(i) Mathews & Lozaityte (1958)	40	90	5	62	23
(i) Buddecke, et al(1963)	5.5		2		16
(ii) Marler & Davidson (1965)	0.75 - 0.9	1.06	1.3	5-6	9.8
(i) Cessi & Bernardi (1965)	2.2		2		14.1
(i) Meyer (1966)	7.5 - 8.0	3-8	1.8	40	7.5
(i) Partridge et al(1961)	7.5	12	2.8	23	16
(i) Partridge (1966)	2.4	1.6	2.8	8-9	6.8
(i) Luscombe & Phelps (1967)	5.4 - 6.7	12-14	2.1	20-25	20
(iii) Muir & Jacobs (1967)	2.3	0.4 - 0.5			?
(i) Serafini-Fracassini et al(1967)	6.3	8.9	2.2 - 2.8	20-24	14.1

(i) for bovine nasal cartilage
(ii) for porcine costal cartilage
(iii) for porcine laryngeal cartilage

as being a proteoglycan and it was considered that each particle represented an individual chondroitin sulphate chain, in coiled conformation, and the intervals between the particles represented the unstained protein core. These dimensions are very similar to those proposed by Partridge, Davis and Adair (1961). They also are in keeping with data derived by Mathews and Lozaityte (1958), especially with respect to the diameter of the molecule and the distance between the chondroitin sulphate chains, but there is disagreement concerning the length of the macromolecule. However, one of the preparations of Mathews and Lozaityte (1958) had a molecular weight, from light scattering, of 1.7×10^6 and a length of 2 900 Å, calculated from root-mean square radius of gyration. In a polydisperse system of rods, such as they were examining, the size of the molecular species under observation is larger than the z-average. (Ehrlich and Doty, 1954). Thus it is likely that the number-average is closer to that indicated by Serafini-Fracassini and Smith (1966).

Some molecular weights for cartilage proteoglycans and their components are shown in Table 2.

It can be seen that there are large variations in both the molecular weights and in the protein to

polysaccharide ratios. Mathews & Lozaityte (1958) pointed out that chondroitin sulphate-protein molecules may form aggregates with molecular weights up to 5×10^7 by lateral and end to end association in which additional non-collagenous protein or ions might participate; while Meyer (1966) suggested that complexes, of above 1×10^6 molecular weight and high protein contents (17%) could be built up of smaller complexes bridged by basic proteins. This hypothesis is supported by several observations. Firstly Hoffman, Mashburn, Meyer & Bray (1967), using preparative electrophoresis, were able to separate the proteoglycan of bovine nasal cartilage into a range of fractions containing from 2.5 to 35.5% protein. Also Partridge, Whiting & Davis (1965) purified a crude proteoglycan preparation (29% protein) on DEAE-cellulose and obtained a chondroitin sulphate-free protein, which had an amino acid composition similar to that of the original complex, and a proteoglycan of molecular weight 2.4×10^5 (Partridge, 1966) containing 7.5% protein. Similarly Serafini-Fracassini, Peters & Floreani (1967) found that precipitation of proteoglycan with bismuth nitrate in acetone reduced the protein content from 18.6 to 14.1%,

while cetylpyridinium chloride precipitation was found to give an intermediate value of 16%. Again, little change was observed in the amino acid composition on purification.

At the other extreme is the proteoglycan extracted from pig laryngeal cartilage by Muir & Jacobs (1967). This had a protein content of only 2%, of which over half the amino acids were either serine or glycine, and a weight-average molecular weight of 230 000.

Possible explanations for the heterogeneity encountered in proteoglycans are variations in the number, size or constitution of carbohydrate chains, in the size of the protein core, and its amino acid composition, in the type of linkage between polysaccharide and protein, or combinations of these. Hoffman et al (1967) consider that the protein core, in the case of nasal and costal cartilage proteoglycan, can have a variable number of polysaccharide chains attached to it and they do not consider that variation in carbohydrate chain length is a probable factor. However, chondroitin sulphates isolated from other tissues have shown variations in molecular weights of 29 000 - 42 000 (horse nasal) (Szirmai, van Boven-de Tyssonsk & Gardell, 1967) and 14 500 - 25 000 (dog epiphyseal) (Hjertquist & Vejlens, 1966).

Heterogeneity of the glycosaminoglycan chains of dermatan sulphate was first reported in 1956, when Hoffman, Linker & Meyer (1956, 1957) showed the presence of both iduronic and glucuronic acids in fractions isolated from pigskin and bullhide, but there have been no reports of discrepancies in the normal disaccharide repeating patterns of chondroitin 4-sulphate and chondroitin 6-sulphate, the major glycosaminoglycan constituents of hyaline cartilage. The fourth possibility is a variation in the length of the protein chain and it can be seen from Table 2 that there appears to be some evidence for this, although some of the molecular weights are calculated and some determinations may represent multiples of a smaller unit through aggregation.

Protein heterogeneity is also suggested by the results of amino end-group analysis, carried out by Serafini-Fracassini, Peters & Floreani (1967) on bovine nasal proteoglycan, where six different α-DNP derivatives were found. This was interpreted to mean that the protein 'core' could be made up of peptide 'sub-units', the sequential arrangement of which was not strictly determined. Further evidence for this theory was supplied when Serafini-Fracassini (1968) found 4 amino-end groups in proteoglycan degraded by

acid-acetone treatment. An alternative explanation might be that there is more than one type of protein. The proteoglycan of Muir & Jacobs (1967) appeared to be a single antigen which cross-reacted with the proteoglycans from other species, but a number of species specific antigens was found in the remainder of the extracted protein-polysaccharide, suggesting that it contained several components. (Loewi & Muir, 1965). However, recent immunologic studies support the view that cartilage contains a family of dissimilar but related proteoglycans (Tsiganos & Muir, 1969 a,b) and in fact cartilage proteoglycan may be separated into a large number of fractions which vary in amino acid composition and with regard to the relative proportions of chondroitin sulphate and keratan sulphate (Schubert & Hamerman, 1968; Tsiganos & Muir, 1970; Hoffman & Mashburn, 1970).

Another type of heterogeneity exists in glycosaminoglycans and has been called Variability by Meyer (1970). This involves differences in the number and sequence of exo groups, such as sialyl, fucosyl and sulphate (Mathews, 1967).

The main protein-glycosaminoglycan linkage of both chondroitin 4-sulphate and chondroitin 6-sulphate apparently contains the seryl-xylosyl-digalactosyl grouping (Lindahl & Rodén, 1966; Helting & Rodén, 1968). However, some heterogeneity of the linkage does exist, as Katsura & Davidson (1966) reported that one out of five chains of chondroitin 4-sulphate of pig costal cartilage was linked via an N-glycosylamine bond to the γ-carboxyl group of glutamate.

In keratan sulphate a high degree of heterogeneity and variation has been demonstrated. This is readily evident from the differences in alkali stability and chemical composition between keratan sulphate I, from cornea, and keratan sulphate II, from cartilage (see Seno, Meyer, Anderson & Hoffman, 1965). Keratan sulphate I is alkali stable and apparently linked via N-glucosaminyl bonds, while keratan sulphate II is alkali labile and linked via O-glycosyl bonds between N-acetylgalactosamine and serine or threonine. It is proposed that the N-acetylgalactosamine group is substituted at carbon 3 by an unknown substituent and that it is linked through carbon 6 to the rest of the carbohydrate chain (Bray, Lieberman & Meyer, 1967).

Patridge, Davis & Adair (1961) and Partridge & Elsden (1961) first suggested that keratan sulphate II was linked to the same protein core as chondroitin sulphate. Although Gregory & Van Lenten (1964) subjected hyaluronidase-degraded proteoglycan to countercurrent fractionation and resolved two components, degraded chondroitin sulphate-protein and undegraded keratan sulphate, apparently attached to a separate protein core, it appears that the current view may best be summarised as follows: chondroitin sulphate proteoglycans do exist which contain no keratan sulphate (e.g. Muir & Jacobs, 1967) but keratan sulphate always occurs together with chondroitin sulphate, although the proportion of keratan sulphate may sometimes be quite high (Pedrini, 1969). The most direct evidence for this relationship came from Seno, Meyer, Anderson & Hoffman in 1965. They demonstrated that a "doublet" fraction, which had been isolated after proteolytic digestion and contained both chondroitin and keratan sulphates, migrated as a single substance in electrophoresis, but that cleavage of the carbohydrate-protein bonds, by treatment with alkali, subsequently permitted separation of the two polysaccharides. They also showed that the chondroitin sulphate was exclusively chondroitin 6-sulphate.

A model, has been proposed by Meyer (1970), for the structure of a proteoglycan containing chondroitin 6-sulphate and keratan sulphate II (Fig. 5). It consists of a double stranded protein backbone with chondroitin 6-sulphate and keratan sulphate II chains starting from either strand. The two strands are crosslinked by keratan sulphate II through another protein-carbohydrate link which is alkali stable (Bray, Lieberman & Meyer, 1967). Another view on the structure of proteoglycans is that of Serafini-Fracassini (1968). The author proposes a basic molecular unit which is composed of three 33 000 molecular weight sub-units, each of which consists of a polypeptide core and two polysaccharide chains. These units are linked together in an end-to-end arrangement. Keratan sulphate II is attached to a separate protein backbone in this model.

As mentioned previously, in brief, one of the factors in the heterogeneity of proteoglycans is variations in the size of polysaccharide chains, i.e. polysaccharide polydispersity. A further examination of this factor is necessary, especially considering different methods of extraction. Molecular weight determinations of chondroitin 4-sulphate from various sources have usually indicated a

moderate degree of polydispersity, although there have been reports of preparations having fairly narrow molecular weight distribution ranges. For example, Mathews (1953) showed that material could be extracted from bovine nasal cartilage that had a weight-average molecular weight of 50 000 and a number-average molecular weight of 40 000. Considering the relatively close agreement between these figures, it was concluded that the degree of polydispersity was slight. However, this glycosaminoglycan had been isolated following digestion by trypsin and chymotrypsin, which is now known to give two polysaccharide chains attached to the same peptide (Seno, Meyer, Anderson & Hoffman, 1965; Luscombe & Phelps, 1967 b; Rosenblum & Cifonelli, 1967; Mathews, 1968). Also, Marler and Davidson (1965) treated proteoglycan, from porcine costal cartilage, with alkali and obtained a chondroitin sulphate preparation which had a weight-average molecular weight of 14 300 and a number-average molecular weight of 13 700, indicating an essentially monodisperse compound.

The fact that different preparations of proteoglycans or polysaccharides may exhibit large variations in their parameters, although prepared by similar methods, has been

recognised for a long time (see Elias & Patat, 1960). There are four possible factors which might account for this during the isolation procedures:-

(a) cleavage of glycosidic bonds by heat

(b) degradation by ascorbate, cysteine or other reducing substances

(c) "mechanical" depolymerisation due to the use of high-speed homogenisers during extraction

(d) the action of lysosomal enzymes.

One effective and commonly used method for tissue solubilisation (Scott,1960) involves digestion with papain in the presence of cysteine and ethylenediamine tetracetic acid at 65 - 70°C. Under these conditions, hyaluronic acid is definitely depolymerised (Blumberg & Ogston, 1957, 1958; Balazs & Sunblad, 1959; Ogston & Sherman, 1959). The proportion of bonds that are broken is small, but even so the molecular weight drops dramatically. Recently, Sajdera & Hascall (1969) compared products obtained after high-speed homogenisation of cartilage, with those obtained by the presumably gentler method of prolonged extraction with 4M. guanidinium chloride or 3M. magnesium chloride. The authors showed that the former method produced material

which was noticeably degraded. However, no definite conclusion can be reached and a more thorough evaluation of preparative procedures is evidently needed in order to distinguish between artifacts arising as a result of degradation during isolation and the true heterogeneity of the proteoglycans in their native state.

The action of lysosomal enzymes during certain extraction procedures is another way in which the native molecule may be changed during isolation (Barrett, 1968; Dodgson & Lloyd, 1968). Several investigators are now employing procedures which minimise the risk of such degradation (e.g. Tsiganos & Muir, 1969 a,b).

PART 1. THE SUB-UNITS OF CHEMICALLY TREATED PROTEOGLYCAN ISOLATED FROM BOVINE NASAL CARTILAGE.

INTRODUCTION

Serafini-Fracassini, Peters and Floreani (1967) suggested that the proteoglycan of bovine nasal cartilage may be made up of sub-units, each consisting of a protein core along which several polysaccharide chains were distributed. This hypothesis was based on the fact that groups of amino acids, in approximately equimolar concentrations, could be detected, together with six amino-end groups, in the protein moiety of highly purified proteoglycan.

Electron-microscopic studies were carried out on proteoglycan preparations by Serafini-Fracassini and Smith (1966) using a bismuth nitrate staining method. They showed that fragmentation can occur along the core of the macromolecule under conditions in which proteolytic degradation cannot be invoked. To investigate this fragmentation under controlled conditions, Serafini-Fracassini (1968) split the proteoglycan of bovine nasal cartilage by

treatment with 0.1M hydrochloric acid in acetone. The treated material was shown, by ultracentrifugal analysis, to consist of two fragments characterised by sedimentation coefficients of 2.3S and 8.1S respectively. The chemical composition of the material remained essentially unchanged, but a reduction in the concentrations of several amino acids, especially tyrosine, was noted. Amino-end group analysis showed the existence of four N-terminal amino acids: aspartic acid, valine, leucine and isoleucine, in a molar ratio of 1:1:0.7:0.3. A number-average molecular weight of 41 000 was derived from this. The author cleared the treated proteoglycan with N-bromosuccinamide and found that the protein content was reduced by 30%. Also, the 8.1S fragment was no longer detectable and the whole material behaved, on ultracentrifugation, as a single 2.2S peak with a number-average molecular weight of 33 000 and a xylose content in agreement with the presence of two polysaccharide chains. A model was proposed in which the molecular unit of the proteoglycan was composed of three 33 000 molecular weight sub-units, each consisting of a polypeptide core and two polysaccharide chains. It was suggested that these molecular units were linked together in an end to end arrangement to form the native proteoglycan macromolecule.

The object of the following investigation was to isolate the two macromolecular species, identified by ultracentrifugal analysis, and to determine their physico-chemical parameters and compositions.

MATERIALS AND METHODS

Isolation of cartilage proteoglycan

Chondromucoprotein was extracted from fresh bovine nasal septa by the procedure of Malawista & Schubert (1958), using low-speed homogenization, to reduce "mechanical" depolymerisation, and cooling the suspension in a low-temperature bath to prevent the temperature rising above 4°. The extract was then fractionated by high-speed centrifugation according to Gerber, Franklin & Schubert (1960). The proteoglycan was precipitated from the supernatant by addition of 2 vol. of ethanol, redissolved in 0.5 M-KCl and centrifuged at 78 000 $g_{av.}$ for 1 hr. All manipulations were carried out as quickly as possible at 4° to avoid proteolytic degradation. After alcohol precipitation, the protein-polysaccharide complex was dissolved in 30% (v/v) ethanol and an aqueous saturated solution of $CaCl_2$ was slowly added while the solution was vigorously stirred. When a strong turbidity developed, 1 vol. of ethanol was added and the precipitate collected by centrifugation, washed with ethanol, dried and stored under reduced pressure over P_2O_5.

Acid acetone treatment and alcohol fractionation

About 10g of the material was dissolved in distilled water, centrifuged at 78 000 $g_{av.}$ for 1 hr., to remove impurities, and precipitated by addition of ethanol (2 vol.) and potassium acetate (10g.). The precipitate was washed exhaustively with anhydrous ethanol, then suspended in 500ml of 0.1M HCl in acetone. The mixture was shaken for 1 hr. at room temperature. The supernatent was filtered off and the cake was washed with acetone to remove excess HCl, then sucked dry on the funnel. (Serafini-Fracassini, 1968).

The acetone-treated material was dissolved in distilled water and the pH adjusted to 7. Ethanol was then slowly added to give a final concentration of 75% (v/v), while the solution was vigorously stirred. The precipitate that formed over 12 hr. was removed by centrifugation at 8 000 $g_{av.}$ for 30 min. and discarded. The ethanol concentration of the supernatant was increased to 85% (v/v) and the precipitated fraction recovered by filtration on a teflon filter and dried in a desiccator over P_2O_5.

Analytical procedures

Hydrolyses for amino acid analysis were carried out in sealed tubes, under N_2, with constant-boiling HCl

(2 ml./mg. of material) at 110° for 24 hr. Acid was removed from the hydrolysates in a rotary film evaporator at 30°. Amino acid analyses were carried out with a Technicon Auto-Analyser. Since the samples contained large amounts of hexosamines, it was found necessary to change the buffer gradient to avoid galactosamine merging with valine. This was done by lowering the pH of the first buffer from 2.875 to 2.750 and by adding to the first two chambers of the Autograd 5 ml. and 3 ml. respectively of methanol.

Amino end-group analysis was carried out by the 2-chloro-3, 5-dinitropyridine method (Signor, Biondi, Terbojevich & Pajetta, 1964). Samples (20-30 mg each) were dissolved in 5 ml. of water in the reaction vessel of a Radiometer pH-stat set at pH 9.0. The microsyringe was filled with 0.25M NaOH and nitrogen was bubbled through the solution agitated with a stirrer. After the pH had stabilised, 6 ml. of ethanol, containing 6.1 mg. of 2-chloro-3, 5-dinitropyridine, were added and the reaction was allowed to proceed for 5 hr. at room temperature. The solution was then acidified to pH2 with HCl and the excess reagent and 2-hydroxy-3, 5-dinitropyridine were removed by several

extractions with ethyl acetate. The dinitropyridyl materials were then precipitated by the addition of 3 vol. of ethanol and potassium acetate, and washed exhaustively with ethanol. The hydrolysis was carried out with 15ml. of constant-boiling HCl in sealed tubes under N_2 at 60^o for 10 hr. The hydrolysate, once brought to pH 2 with KOH, was extracted with six portions (10 ml. each) of ethyl acetate. The pooled ethyl acetate extracts were washed twice with 5ml. of 0.01N-HCl. The water phase together with the acid washings of the ethyl acetate extracts were taken to dryness and the residue was dissolved in constant-boiling HCl and hydrolysed, in sealed tubes under N_2, at 110^o for 24 hr. prior to amino acid analysis.

For hexosamines, samples of known weights (approx. 1 mg.) were dissolved in 2ml. of 4.5N-HCl and hydrolysed in sealed tubes, under N_2, at 105^o for 8 hr. After hydrolysis, excess of acid was neutralised and total hexosamine was determined by the Elson & Morgan (1933) reaction using the distillation procedure of Cessi & Pliego (1960). Differential determination of glucosamine and galactosamine was carried out by column chromatography (Partridge & Elsden, 1961).

Hexuronic acid was estimated directly on unhydrolysed solutions by the method of Bitter & Muir (1962), with glucuronic acid as the standard.

The protein concentration of gel filtration eluates was determined by the micro-biuret method of Itzhaki & Gill (1964).

Gel filtration

A column, 1.5 cm. × 90 cm., was packed, as described by Flodin (1962), with agarose gel obtained from a commercial preparation (Bio-Gel A-15m, 100-200 mesh; Bio-Rad Laboratories, Richmond, U.S.A.). Prior to use, the packed column was eluted with 1M potassium acetate, adjusted to pH 7 with acetic acid, under the conditions of flow rate required for subsequent experiments (9.5 ml./hr.). The final bed volume was 124 ml. The void volume was determined with Narcissus Mosaic Virus and the gel column was calibrated by estimation of the elution volumes of a series of proteins of known Stokes radius. These included: bovine thyroglobulin (Edelhoch, 1960), glutamic dehydrogenase (Rogers, Hellerman & Thompson, 1965), yeast alcohol dehydrogenase (Hayes & Velick, 1954) and horse radish peroxidase (Cecil & Ogston, 1951). Samples of these standards,

dissolved in 1M potassium acetate pH 7, were applied to the column in 1.5 ml. aliquots for zonal analysis. Fractions (1.5 ml. each) were collected, the effluent being continuously monitored at 260 nm with a base-compensating automatic recorder, each fraction being tested for its protein content.

A second column, 2.5 cm. × 60 cm., was packed with 1% agarose (Bio-Gel A-150m, 100-200 mesh; Bio-Rad Laboratories, Richmond, U.S.A.) and equilibrated with 1M potassium acetate pH 7, as described above. The flow rate was adjusted to 12 ml./hr.

Proteoglycan preparations, dissolved in 1M potassium acetate, were applied to the columns in 1.5 ml. loads. Effluent fractions were monitored by determination of their hexuronic acid content.

Viscometry

Viscosity measurements were made at 25° ± 0.01 with a capillary viscometer constructed as described by Fox, Fox & Flory (1951). The rate of shear characteristic of this instrument, when filled with 7 ml. of distilled water, was 661 $sec.^{-1}$ Samples of fractions 1 and 2 (see RESULTS p.31) were dissolved in 0.05M-KCl at a concentration of approx.

0.2 g./100 ml. The pH was then adjusted in the range 2.5 - 7.5 and 7 ml. of each solution were pressure-filtered into the viscometer through a no. 2 sintered glass.

Osmometry

The instrument used was a high-speed osmometer (Hewlett-Packard, Pa., U.S.A.) equipped with B-19 membranes (Schleicher & Schuell, Keene, Germany). The constant temperature control was set at 20°. All osmotic pressure measurements were made with a high concentration of diffusible electrolyte. Each sample was dissolved in and dialysed against 2M-KCl, adjusted to suitable pH with HCl. Dialyses were carried out for 4 days at 4°. Proteoglycan concentrations were determined before and after dialysis by estimation of the hexuronic acid content of the solutions and by differential refractometry. Progressive dilutions of each sample were prepared and at least four osmotic pressure determinations were made for each concentration.

Electron microscopy

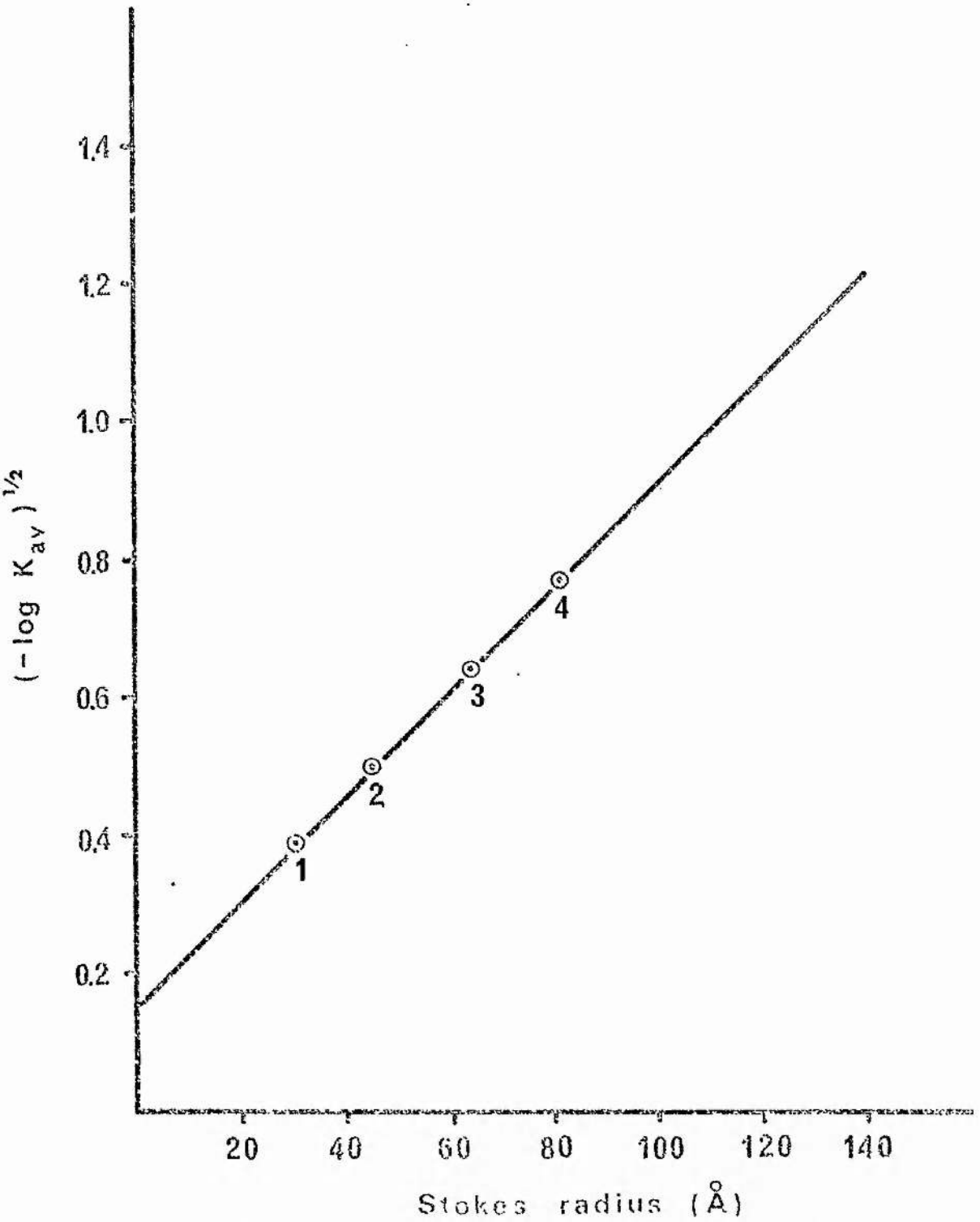
An aqueous solution of acetone-treated proteoglycan was sprayed, at a concentration of approx. 3 µg./ml., on to carbon-coated grids. Once dry, the grids were stained for 10 min. in 0.5% (w/v) bismuth nitrate in 0.1N-HNO_3

(Serafini-Fracassini, Durward & Crawford, 1969). They were then washed in 0.1N-HNO_3, followed by distilled water, and dried under reduced pressure in a desiccator. Micrographs were taken using an AEI EM6B electron microscope.

RESULTS

The gel filtration data obtained for the four proteins chromatographed for the calibration of the 4% agarose column were used in the elaboration of the $(-\log K_{av.})^{1/2}$ versus Stokes radius plot, according to Laurent & Killander (1964), as shown in Fig. 6. The high ionic strength of the buffer used in the elution of the gel column was intended to avoid adsorption phenomena and particularly to reduce the repulsive effects among fixed charges on the glycosaminoglycan chains, in order to maximize the uniformity of their effective hydrodynamic volumes in a random coil conformation. The elution profile of the acetone-treated proteoglycan on the 4% agarose column is shown in Fig. 7. The retarded material, accounting for approx. 55% of the recovered hexuronic acid, was resolved into two fractions having elution volumes of 70 ml. (fraction 1) and 109 ml. (fraction 2). These values were converted, on the basis of the calibration curve shown in Fig. 6, to the corresponding Stokes radii of 134 Å and 47 Å respectively.

Figure 6

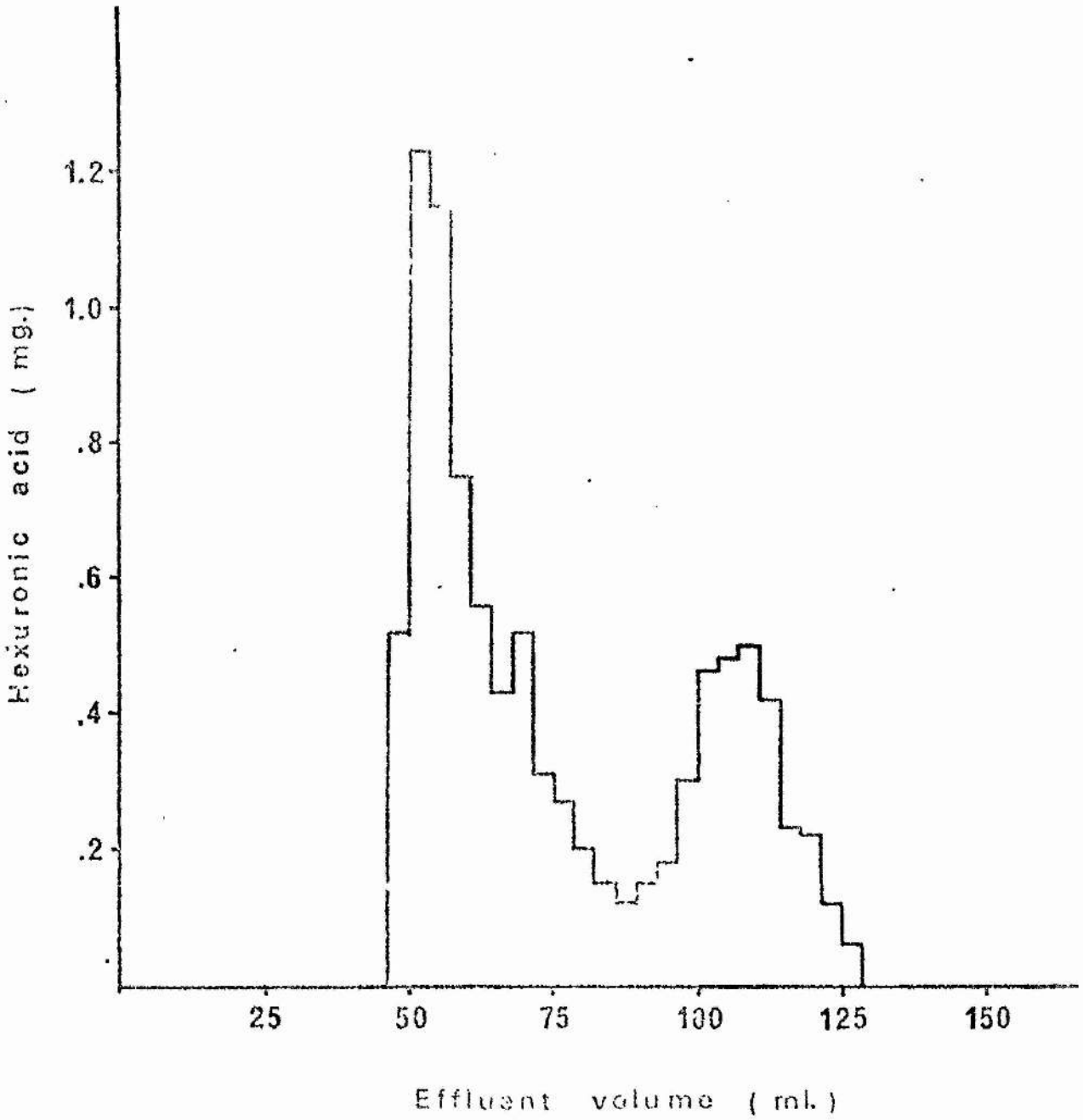


Plot correlating the elution volume and the Stokes radius of four proteins on a 4% agarose column (bed volume 124 ml., void volume 52 ml.) at pH 7. Experimental details are described in the text.

1 - bovine thyroglobulin, 2 - glutamic dehydrogenase,

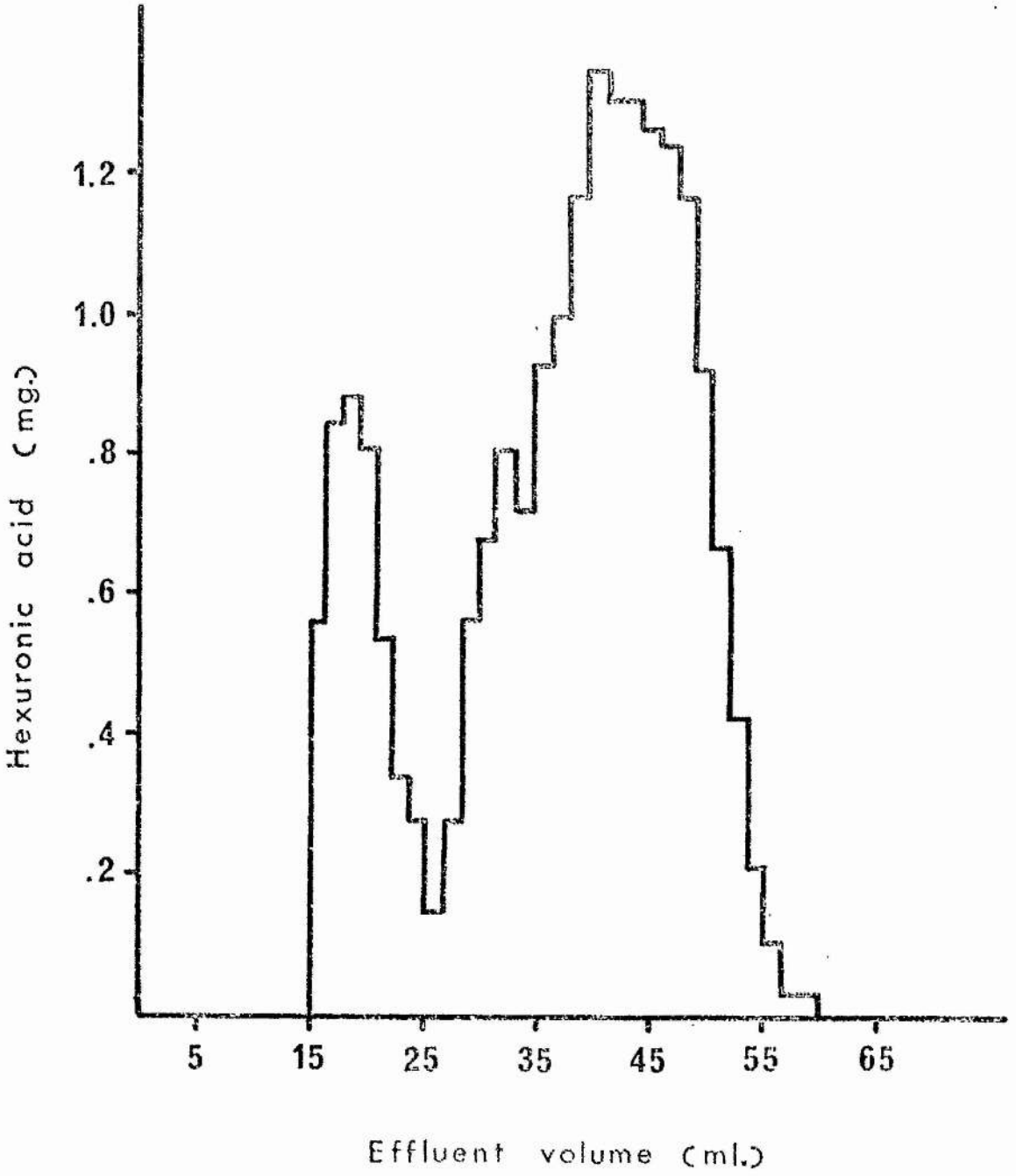
3 - yeast alcohol dehydrogenase, 4 - horse raddish peroxidase.

Figure 7



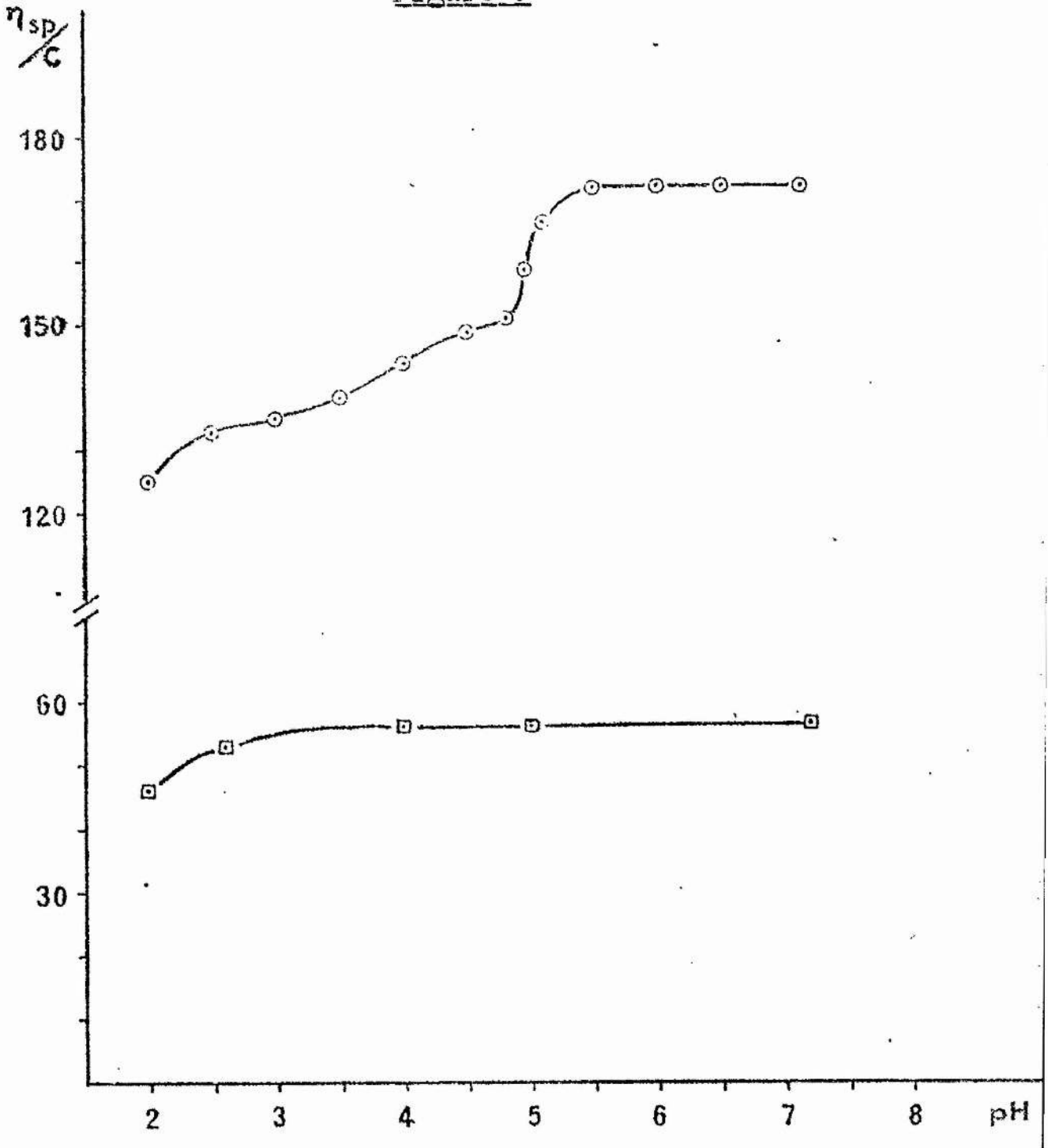
Elution of acid-acetone treated proteoglycan (expressed as hexuronic acid) with 1M potassium acetate, pH 7, from a column of 4% agarose (bed volume 124 ml., void volume 52 ml.)

Figure 8



Elution of acid-acetone treated proteoglycan (expressed as hexuronic acid) with 1M potassium acetate, pH 7, from a column of 1% agarose. Void volume 18 ml.

Figure 9



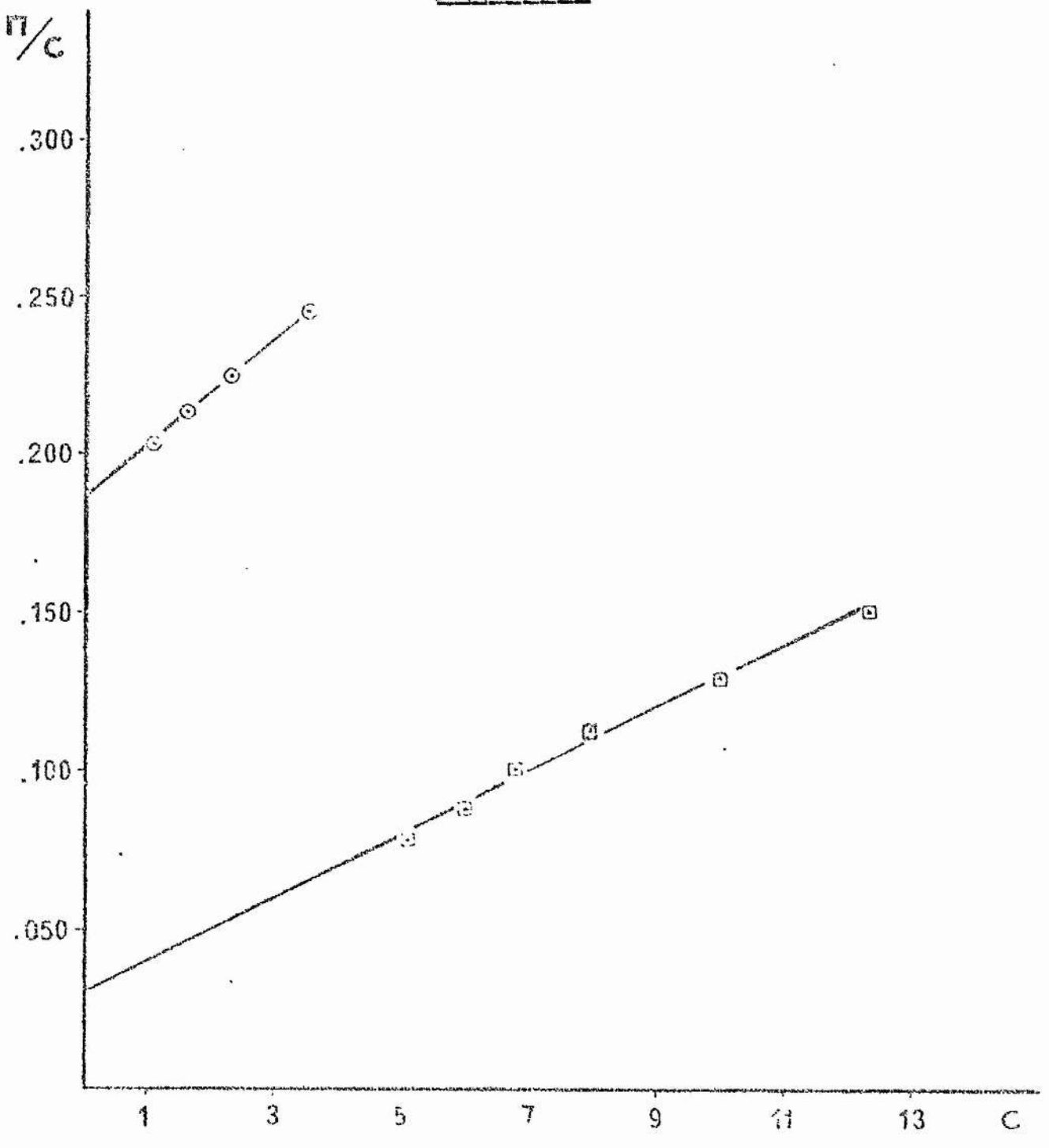
Viscosity number versus pH plots for fraction 1 (⊙) and fraction 2 (⊡) at a glycosaminoglycan concentration of 0.2% in 0.05M-KCl, 25° and 551 $sec.^{-1}$ shear rate.

In order to remove high-molecular weight species contaminating fraction 1, as indicated by analytical ultracentrifugation that revealed a rapidly sedimenting material (Serafini-Fracassini, 1968), a preliminary separation was carried out on the 1% agarose column and a bimodal elution pattern was obtained (Fig. 8). The whole of the second peak (ml. 28 to 55) was rechromatographed on 4% agarose and a similar pattern to that reported in Fig. 7 was obtained, although the excluded material was much reduced.

Fraction 1 (ml. 65 to 85) and fraction 2 (ml. 95 to 120) were isolated from the eluates of a series of chromatographic separations on 4% agarose after prior purification on 1% agarose.

The viscosity of the two fractions was determined at different hydrogen ion concentrations. Fig. 9 shows such plots of reduced viscosity versus pH. KCl 0.05M was used as the solvent, this being an ionic concentration at which the viscosity of the proteoglycan has been shown to be independent of the amount of salt present in solution (Luscombe & Phelps, 1967[a]). While fraction 2 gave a normal curve over the pH range examined, fraction 1 showed an

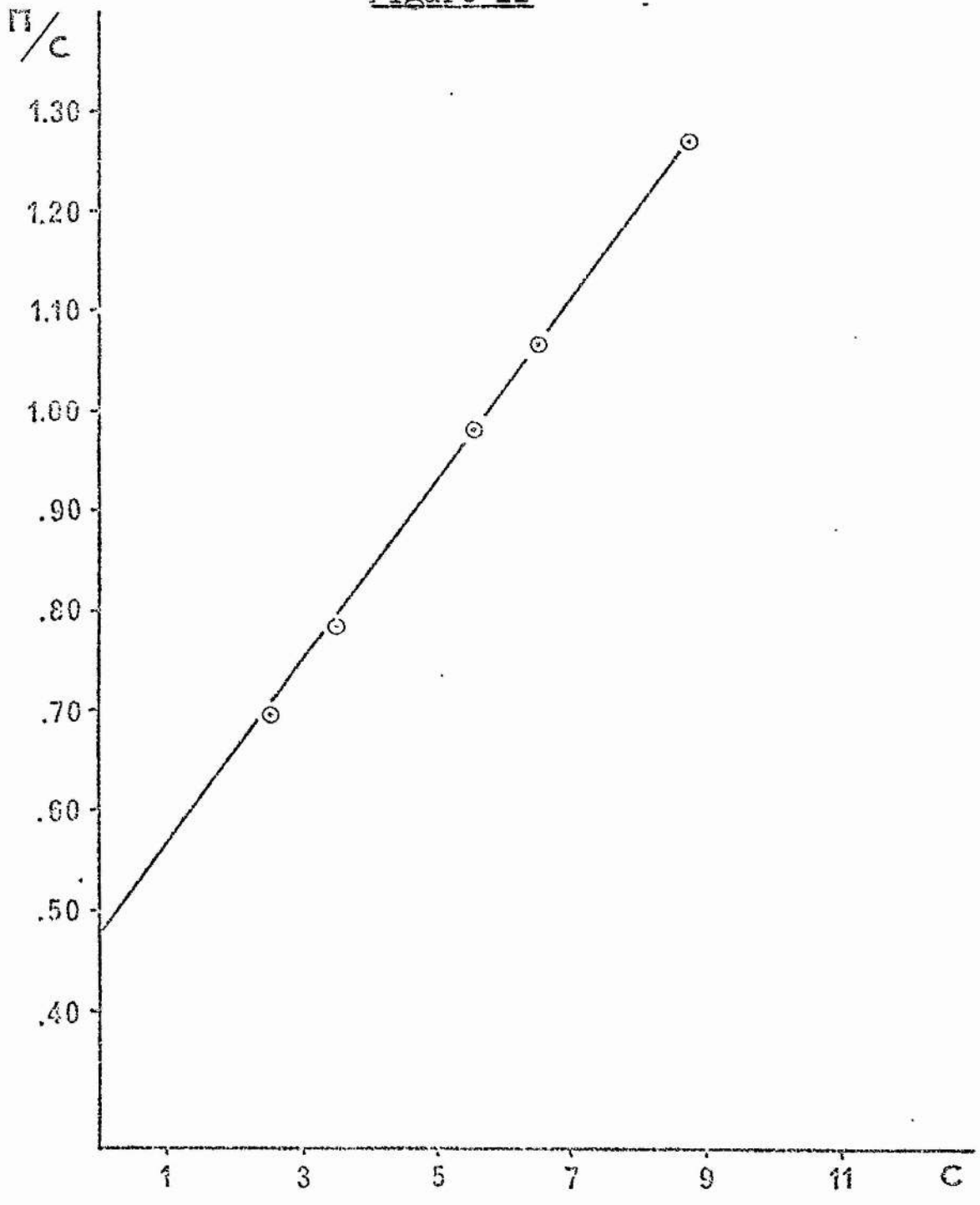
Figure 10



Plots of π/c versus c obtained for fraction 1 in 2M-KCl at pH 3.5 (⊙) and pH 7 (⊡).

10

Figure 11



Extrapolation of the reduced osmotic pressure of fraction 2 in 2M-KCl, pH 7.

Plate 1

Monolayer of acid-acetone treated proteoglycan sprayed on to a carbon-coated grid at a concentration of 3 µg./ml. Bismuth nitrate staining.

Arrows a indicate long beaded filaments composed of several 30 Å particles. Arrows b point to short beaded segments composed of three 30 Å particles, while arrows c point to isolated particles.

plate I

Table 3. Amino acid analyses

Values are expressed as µg. of anhydro-amino acid per 10 mg. of ash- and moisture-free samples.

Sample	Fraction 1	Fraction 2
Amino acid		
Hyp	0.0	0.0
Asp	226.1	37.4
Thr	110.1	23.0
Ser	154.2	39.3
Glu	343.4	71.8
Pro	139.5	37.7
Gly	124.0	38.3
Ala	91.0	23.0
Val	129.5	12.8
Cys	0.0	0.0
Met	0.0	0.0
Ile	77.1	12.1
Leu	179.5	29.0
Tyr	56.8	11.4
Phe	115.1	18.2
Hyl	0.0	0.0
Lys	76.3	11.3
His	37.1	0.0
Arg	140.3	26.7
Totals	2000.0	392.0

anomalous curve with a major inflection point at pH 4.9.

Concurrently, molecular weight determinations were carried out by osmometry on both fractions at pH 7. An additional molecular weight determination of fraction 1 was performed at pH 3.5 as the hydrogen ion concentration appeared to affect the physico-chemical parameters of this macromolecular system in solution. The plots of the reduced osmotic pressures (π/c) as functions of solute concentrations (c), reported in Fig. 10, are consistent with a number average molecular weight of 750 000 for fraction 1 at pH 7 and with one of 122 500 for the same fraction at pH 3.5. A molecular weight of 47 400 was calculated for fraction 2 (Fig. 11).

Plate 1 shows a typical field of the monolayer produced by spraying the acetone-treated proteoglycan on carbon-coated grids. Together with long beaded filaments (arrows a), interpreted as polymeric forms, both short segments, composed of three 30 Å particles, (arrows b) and isolated particles (arrows c) can be seen.

The amino acid compositions of fraction 1 and fraction 2 are reported in Table 3. Each amino acid concentration was corrected for hydrolytic losses by

Table 4. Chemical analyses

Values are expressed as percentages of dry ash-free samples.

Sample	Fraction 1	Fraction 2
Protein	20.0	3.9
Total hexosamine (as free base)	23.8	32.1
Glucosamine to galactosamine ratio	1:4.8	1:23.1

Table 5. End-group analysis of Fraction 2

Values are expressed as moles of amino acid per 10^6 g. of sample.

Ile	9.0
Leu	15.8
Total	24.8

applying the coefficients reported in a previous paper (Serafini-Fracassini et al. 1967). The protein contents, obtained by summation of these corrected values, are reported in Table 4 together with the results of all other chemical analyses.

The results of the amino end-group determination of fraction 2, reported in Table 5, show two N-terminal amino acids. The molecular weight calculated from the total number of moles of N-terminal amino acids/10^6 g. of proteoglycan is 40 300, which is in keeping with the value obtained by osmometry on the same fraction.

DISCUSSION

The combined information gathered from analytical gel filtration and ultracentrifugation (Serafini-Fracassini, 1968) indicates that the acetone-treated proteoglycan preparation examined is composed of a high-molecular weight fraction and of two discrete macromolecular species.

The smaller of these two macromolecular species, which behaves as a single homogeneous fraction (fraction 2) during gel filtration on 4% agarose and which corresponds to the fragment characterized by a sedimentation coefficient of 2.3 S, has a number average molecular weight 47 400. Its effective hydrodynamic volume is expressed by a Stokes radius of 47 Å in 1M potassium acetate. The other macromolecular fragment, which is eluted during gel filtration on 4% agarose as fraction 1, exhibits a Stokes radius of 134 Å and 122 500 mol.wt. at pH 3.5. These physico-chemical parameters would suggest that the latter macromolecules are constituted of three molecules similar in size to the former units and assembled together by an end-to-end alignment of their core polypeptides. On the basis of this size relationship only, the 47 Å-Stokes

radius fragment will be referred to as the monomer and the 134 Å component as the trimer.

It is worth noticing, however, that the compositions of the two macromolecular species reveal large differences. The monomer has a protein content of 4% and a very low glucosamine concentration which is probably attributable to a contamination of keratan sulphate. The trimer, on the other hand, has a much higher protein content (20%) and a glucosamine to galactosamine ratio of 1:4.8. Serafini-Fracassini (1968) showed the number-average molecular weight of the chondroitin sulphate chain of a similar acetone-treated proteoglycan preparation, cleaved by N-bromosuccinimide, to be approximately 19 000 by osmometry. Therefore the monomer appears to be constituted by two chondroitin sulphate chains linked by a short polypeptide. When the amino acid concentrations of this polypeptide are expressed as number of amino acid residues per 47 000 mol.wt. unit, 2.1 residues of serine are found to be present and presumably engaged in the glycosidic linkage with the glycosaminoglycan chains. Of the four N-terminal amino acids previously detected in the acetone-treated proteoglycan (Serafini-Fracassini, 1968), only

two are present in the monomer indicating that at least two different core polypeptides are associated with chondroitin sulphate in bovine nasal cartilage.

If it is assumed that all glucosamine were derived from keratan sulphate, having a chain weight of approx. 10 000 (Mathews & Cifonelli, 1965), the molecular weight of the trimer and its glucosamine content would indicate the presence of four chondroitin sulphate and two keratan sulphate chains in the same macromolecular unit. The high protein content of the trimer could then be attributable to the keratan sulphate moiety, this being in keeping with the findings of Hoffman, Mashburn, Meyer & Bray (1967) and Hoffman, Mashburn & Meyer (1967).

Although the presence of keratan sulphate does not prevent the core of the proteoglycan from being split by the acid-acetone treatment, it seems to stabilize the structure, in which it is involved, to the extent of avoiding its complete degradation to monomeric forms.

It is worth noticing that fraction 1, comprising of the trimer, has a molecular weight of 750 000 as determined by osmometry under conditions similar to those used in gel filtration. Such a value is not in agreement with

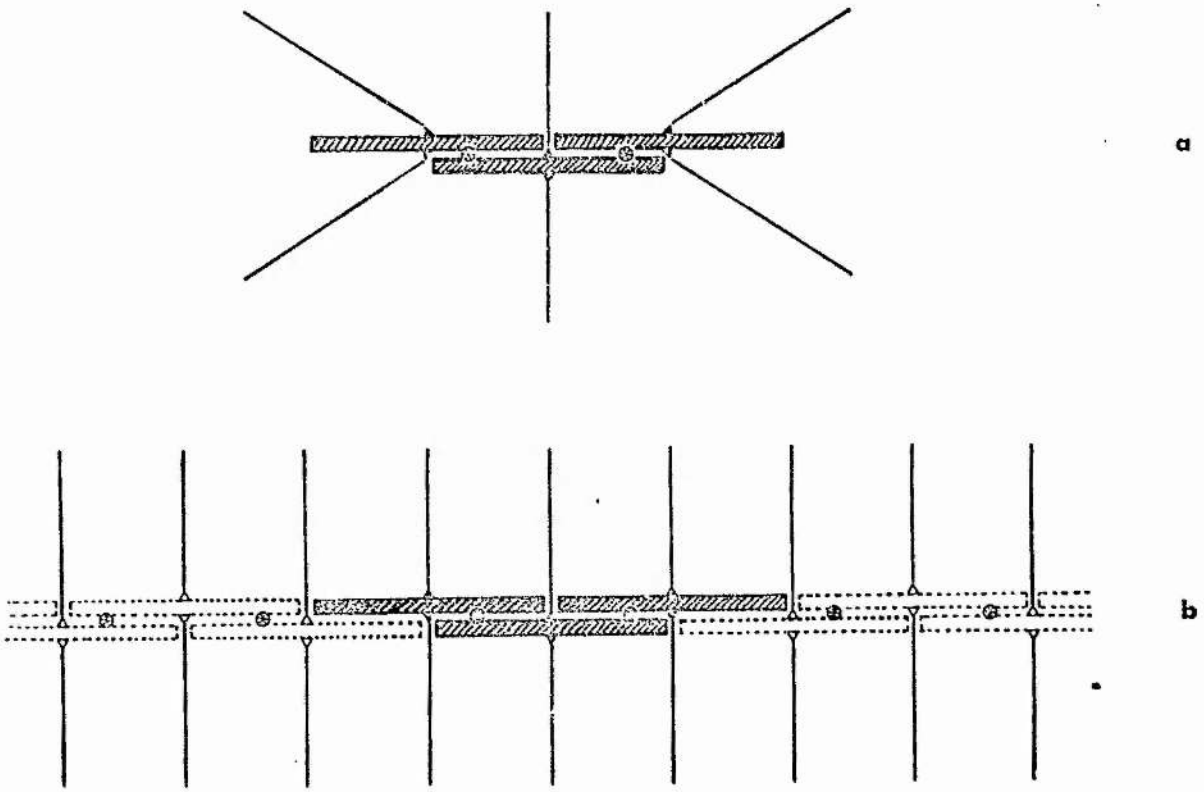
that which is expected considering the Stokes radius of the macromolecule, if a comparison between fractions 1 and 2 can be drawn. However, when osmometric determinations are carried out at pH 3.5, the values for molecular weight and hydrodynamic volume are consistent. This can be interpreted as being due to the ability of the trimer to undergo aggregation at neutral pH in spite of the high ionic strength of the buffer used in both experiments. If this hypothesis is correct, fraction 1, as shown in Fig. 7, is constituted by isolated trimer molecules in equilibrium with aggregates excluded by the agarose gel. The isolation of fraction 1 alters this equilibrium, thus resulting in aggregation of free trimer molecules until a new equilibrium is attained, unless the process is prevented by lowering the pH.

The viscosity experiment carried out on fraction 1 not only substantiates this view but proves the reversibility of the aggregation as the result of a change in the hydrogen ion concentration of the system, with a fairly sharp transition point between association and dissociation at pH 4.9. The increase in asymmetry of the macromolecules, revealed by the rise in viscosity of

the solution, could be interpreted as being due to an end-to-end association of several trimer molecules. A similar effect of solvent pH on macromolecular aggregation has been reported by Hascall & Sajdera (1969) in their study of a proteoglycan fraction, characterized by a sedimentation coefficient of approx. 20 S, isolated from bovine nasal cartilage. These authors demonstrated that aggregation of the 20 S sub-units is initiated by non-covalent interaction between proteoglycan macromolecules and a specific glycoprotein. It is interesting to note that such a phenomenon is occurring both in systems composed of high-molecular weight complexes and in solutions of low-molecular weight proteoglycans, as shown in the present study. The sharp inflection point, at pH 4.9, in the viscosity curve of the trimer indicates that probably complete ionization of the carboxylic groups is necessary for aggregation to occur.

The existence of the trimer as a macromolecular entity is further supported by the electron micrograph, shown in Plate 1, in which short segments composed of three electron-dense particles are evident. The specificity of Bi^{3} for glycosaminoglycans, under the conditions

Figure 12



Idealised model of trimer

a. Trimer in free form. Scored bar, polypeptide core; circle, inter-monomer bonds; triangle, xylosyl-serine link; line, polysaccharide chain.

b. Trimer incorporated into the proteoglycan macromolecule. Symbols as above.

used during the staining of the grids, has been shown and a correlation between each 30 Å-particle and a doublet of polysaccharide chains has been made (Serafini-Fracassini and Smith, 1966; Smith, Peters and Serafini-Fracassini, 1967; Serafini-Fracassini, Durward and Crawford, 1969). Moreover, in this electron micrograph the 60 Å period along the segments is still present which is in keeping with earlier observations on the native proteoglycan (Serafini-Fracassini & Smith, 1966) and with the Mathews & Lozaityte (1958) model for chondromucoprotein.

In Fig. 12, models for the trimer both in free and polymerised form are shown. If the arrangement depicted in the diagram is repeated consistently along the macromolecule, a double-stranded protein core would result. This is in conformity with previous observations regarding the molecular weight, 89 000 (Serafini-Fracassini, Peters and Floreani, 1967) and length, 1,100 - 1,500 Å (Serafini-Fracassini and Smith, 1966), of the protein core of a proteoglycan preparation. An average amino acid residue weight of 105 indicates that such a protein core is composed of about 850 amino acids. Assuming an axial translation of 3.3 Å per amino acid residue, the length

of a single chain core would be approximately 2,800 Å. Thus a triple chain, or more especially a double-chain core would be more in keeping with the macromolecular length of 1,100 - 1,500 Å than one consisting of a single chain of amino acids.

The isolation of two distinct macromolecular species after acid-acetone treatment of cartilage proteoglycan implies the existence of labile bonds periodically distributed along the core of the native complex. Although the material examined underwent chemical treatment, this study suggests that monomer and trimer could represent true sub-units of the protein-polysaccharide complex of bovine cartilage.

SUMMARY

1. The light fraction of the proteoglycan of bovine nasal cartilage was split by treatment with 0.1N-HCl in acetone. The products were separated by gel filtration on 4% agarose and two retarded fractions were detected and isolated. These two fractions were found to have a Stokes radius of 134 Å and 47 Å respectively, as determined by calibration of the column against proteins of known hydrodynamic volumes.

2. The 47 Å-fraction had a protein content of 4% and a glucosamine to galactosamine ratio of 1:23. The 134 Å-fraction had a protein content of 20% and a glucosamine to galactosamine ratio of 1:4.8.

3. Viscometric studies on both fractions showed that the 134 Å-fraction alone exhibited the property of undergoing reversible, pH-dependent aggregation with a transition point at pH 4.9.

4. It was concluded that these fractions could represent sub-units of the native cartilage proteoglycan.

PART 2. THE IDENTIFICATION, IN VIVO, OF THE MACROMOLECULAR SUB-UNITS OF CARTILAGE PROTEOGLYCAN.

INTRODUCTION

It was suggested in Part 1 that the native proteoglycan of bovine nasal cartilage is composed of several sub-units assembled together in a linear arrangement and linked through acid-labile bonds.

If this hypothesis is correct, chondrocytes would synthesize in their endoplasmic reticula, glycosaminoglycan-peptides of low molecular weight. These could then be directly released into the extracellular matrix where polymerisation would take place. Conversely, polymerisation could occur intracellularly, geared to the biosynthesis of the various components, the product being excreted in the form of a high molecular weight macromolecule.

In Part 2, the molecular weight distribution patterns of glycosaminoglycan-containing complexes, extracted from rabbit ear cartilage, were examined to ascertain whether low molecular weight macromolecules, similar to those obtained by chemical means, could be identified as intermediates of proteoglycan biosynthesis. To facilitate

this study, use was made of the so called "donkey phenomenon" of rabbit ears, which can be induced by the administration of crude papain.

In 1956, Thomas reported that an intravenous injection of crude papain in rabbits caused, within a few hours, loss of the normal rigidity of cartilage to such an extent that the ears of the animals collapsed. Cartilaginous tissues in all parts of the body were found to have lost their normal degree of basophilia and metachromasia, and, in the ear cartilage, much of the actual substance of the matrix disappeared, leaving a shrunken cartilage plate with normal appearing chondrocytes packed side by side. Within two or three days, the matrix was restored, and the ears were again upright.

Apart from the change in cartilage matrix, no other lesions were encountered in these animals, although Kellner and Robertson (1954), using larger doses, had previously described necrotizing changes involving the myocardium following intravenous papain. The only evidence of inconvenience to the animals was respiratory difficulty in some, attributable to partial collapse of the softened trachea and bronchial tubes. No histological evidence

of damage or loss of metachromasia in connective tissue other than cartilage matrix was found.

The disappearance of metachromatic matrix became evident in the cartilage plates of ear and trachea within 3 or 4 hours after papain, but the epiphyseal plates, which seemed much more vulnerable to the effect, were found by Westerborn (1961) to show depletion as early as 10 minutes after injection.

Biochemical evidence for the release of chondroitin sulphate from cartilage into the circulating blood was obtained by employing direct assays for glycosaminoglycans in the blood or urine, or measuring the release of ^{35}S from previously labelled cartilage (Bryant, Leder & Stetten, 1958; Tsaltas, 1958; Potter, McCluskey, Weissmann & Thomas, 1960). Radioautographs of ear cartilage showed depletion of ^{35}S within 4 hours after papain.

The active principle in papain was found to be the crystallizable papain proteinase originally isolated by Kimmel & Smith (1957). Experiments with purified proteinase failed to produce the expected phenomenon but if the enzyme were injected in its oxidised, inactive state it was shown to be highly active on cartilage (McCluskey & Thomas, 1958).

It was also rapidly effective when inactivated by dialysis or by the addition of anti-thiol substances such as parachloromercuribenzoate. Findings suggest that activated papain is prevented from reaching cartilage by it becoming rapidly bound to serum proteins, perhaps as substrate, and thus it fails to leave the circulation (Potter et al, 1960). There also must exist a reducing system of some sort in cartilage to restore the activity of inactive papain once it has gained access to the tissue.

Thus, there is available an experimental model in which a potent protease can circulate in the blood in its activated form and cause no tissue damage, while the inactive (and therefore enzymatically undetectable) form of the same enzyme can produce, selectively, widespread disintergration of a particular tissue.

The inhibiting action of cortisone on the synthesis of glycosaminoglycans in connective tissue has been studied by several investigations. Layton (1951) found that cortisone reduced the fixation of ^{35}S in the skin of rats; this was confirmed by Boström & Odeblad (1953), who found the same effect in costal cartilage. It was later shown that the inhibition of sulphate incorporation was based on

impaired synthesis of the entire glycosaminoglycan molecule (Schiller & Dorfman, 1957).

When cortisone was administered daily following a single injection of papain it was found that the tissue remained in its depleted state for as long as four weeks (Thomas, 1956; McCluskey & Thomas, 1959). This inhibition was demonstrated to be due to a direct action of cortisone on cartilage. Intra-articular injections of small doses of prednisolone prevented recovery of the articular cartilage in that joint, and also inhibited the local uptake of ^{35}S without affecting the cartilage in other areas (McCluskey & Thomas, 1959).

MATERIALS AND METHODS

Treatment of animals for experiments and preparation of cartilage

A group of 40 New Zealand albino rabbits, 6-8 weeks old and each weighing less than 1 kg., were injected in the marginal ear vein with 1 ml. of a 2% (w/v) aqueous solution of crude papain (Thomas, 1956). Bacteria-free papain solutions were obtained by filtration through 50 mμ filters. Cortisone acetate was then administered intramuscularly, in the dose of 5 mg./kg. body weight (Thomas, 1956), immediately after the papain injection and thereafter at 24 hr. intervals. This cortisone treatment was required to suppress cartilage proteoglycan biosynthesis (Asboe-Hansen, 1954; Thomas, 1956; McCluskey & Thomas, 1959) until complete elimination of degraded proteoglycans, produced by papain action (Bryant, Leder & Stetten, 1958; Muir, 1958; Tsaltas, 1958; Weissman, Potter, McCluskey & Schubert, 1959) was achieved. Therefore, concurrent experiments were carried out to assess the time required for the serum glycosaminoglycan content, increased by the papain-induced loss of cartilage

components into the blood stream (Bryant et al. 1958; Muir, 1958; Tsaltas, 1958; Weissman et al. 1959; Crosti, Catchpole & Pirani, 1963), to return to control levels. For this purpose, 3 groups of 3 New Zealand albino rabbits were used. The first group was kept as a control. The rabbits in the second group were injected intravenously with a 2% (w/v) solution of albumin and those in the third group with papain, as described above. All three groups were then treated with cortisone acetate at 24 hr. intervals. The rabbits were bled before albumin or papain administration and thereafter every 24 hr. Sera were analysed for glycosaminoglycan content by the method of Weissman et al. (1959).

Since this experiment indicated that five days were sufficient to restore the serum glycosaminoglycan level to normal values (see RESULTS, Fig.13), the cortisone treatment of the 40 papain-treated animals was discontinued after this time and the rabbits were sacrificed, in groups of ten, at respectively 36 hr., 40 hr., 48 hr. and 72 hr. after the last cortisone injection.

The ear cartilage, quickly freed from surrounding tissue, was dried in cold acetone and ground to a fine powder.

Thirty rabbits were treated with papain and cortisone as described above. Each animal was then injected intraperitoneally with 2 mc of radiosulphate, as $Na_2{}^{35}SO_4$ in water, 40 hr. after the last cortisone administration. The animals were then sacrificed, in groups of ten, after 30 min., 1 hr. and 5 hr. and the ear cartilage was removed, dried and ground.

Isolation and purification of cartilage proteoglycan

The proteoglycan was extracted from the acetone-dried powder of ear cartilage essentially as described by Malawista & Schubert (1958), but using low-speed homogenization. The cartilage suspension in distilled water (5 g. in 200 ml.) was cooled in a low-temperature bath and extraction was carried out for a few minutes at a time, for a total of 1 hr., to prevent the temperature rising above 4°. The fine homogenate was centrifuged at 2 000 g for 1 hr. and the supernatant fractionated by high-speed centrifugation (Gerber, Franklin & Schubert, 1960). The proteoglycan contained in the supernatant was collected by ethanol precipitation and stored under reduced pressure over P_2O_5.

The cartilage fragments, remaining after proteoglycan extraction, and the residue of the high-speed centrifugation were pooled, dried and re-extracted as follows. Aliquots, 10 g. each, were suspended in 400 ml. of 2M-$MgCl_2$ containing 1% (w/v) cetylpyridinium chloride and homogenized at 50° for 1 hr. After centrifugation at 38 000 $g_{av.}$ for 1 hr. at 40°, the clear supernatant was diluted with distilled water to give a concentration of 0.6M-$MgCl_2$ (Scott, 1960). The precipitate that formed during 12 hr. was collected by filtration on a cellulose filter pad. The pad was washed with 0.6M-$MgCl_2$ and then eluted with 200 ml. of 2M-$MgCl_2$ at 40°. Cetylpyridinium chloride was dissociated from the complex and extracted by shaking the solution with 5 vol. of chloroform—n—pentan-1-ol (5:4, v/v) (Scott, 1960) at room temperature. The complete removal of cetylpyridinium chloride from the aqueous phase was shown spectrophotometrically by the disappearance of its characteristic E^{o}_{260}. Five volumes of ethanol were then added to the solution and the proteoglycan collected by centrifugation, washed with acetone, dried and stored in a desiccator over P_2O_5.

Chemical assays

The hydrolysis for hexosamine analysis was carried out in 4.5N-HCl (2 ml./mg. of material) at 100° for 8 hr. under N_2 in sealed tubes. Excess of acid was neutralized and total hexosamine was determined by the method of Cessi & Piliego (1960).

Quantitative determination of hexuronic acid was performed by the method of Bitter & Muir (1962), with glucuronic acid as the standard.

Sulphur was determined by the method of Giellman & Tölg (1960).

Total hexoses were estimated by the method of Scott & Melvin (1953).

Quantitative determination of protein was carried out by the micro-biuret method of Itzhaki & Gill (1964).

Infrared absorption

Infrared absorption spectra of the samples were obtained on a Unicam 200G infrared recording spectrophotometer, using Nujol mulls.

Gel filtration

The two columns, already described in Part 1, were used. Samples (20 mg.) were dissolved in 1.5 ml. of

1M potassium acetate and 3 ml. fractions were collected from the columns. The effluent fractions were monitored for proteoglycan by determination of their hexuronic acid content.

Liquid scintillation counting

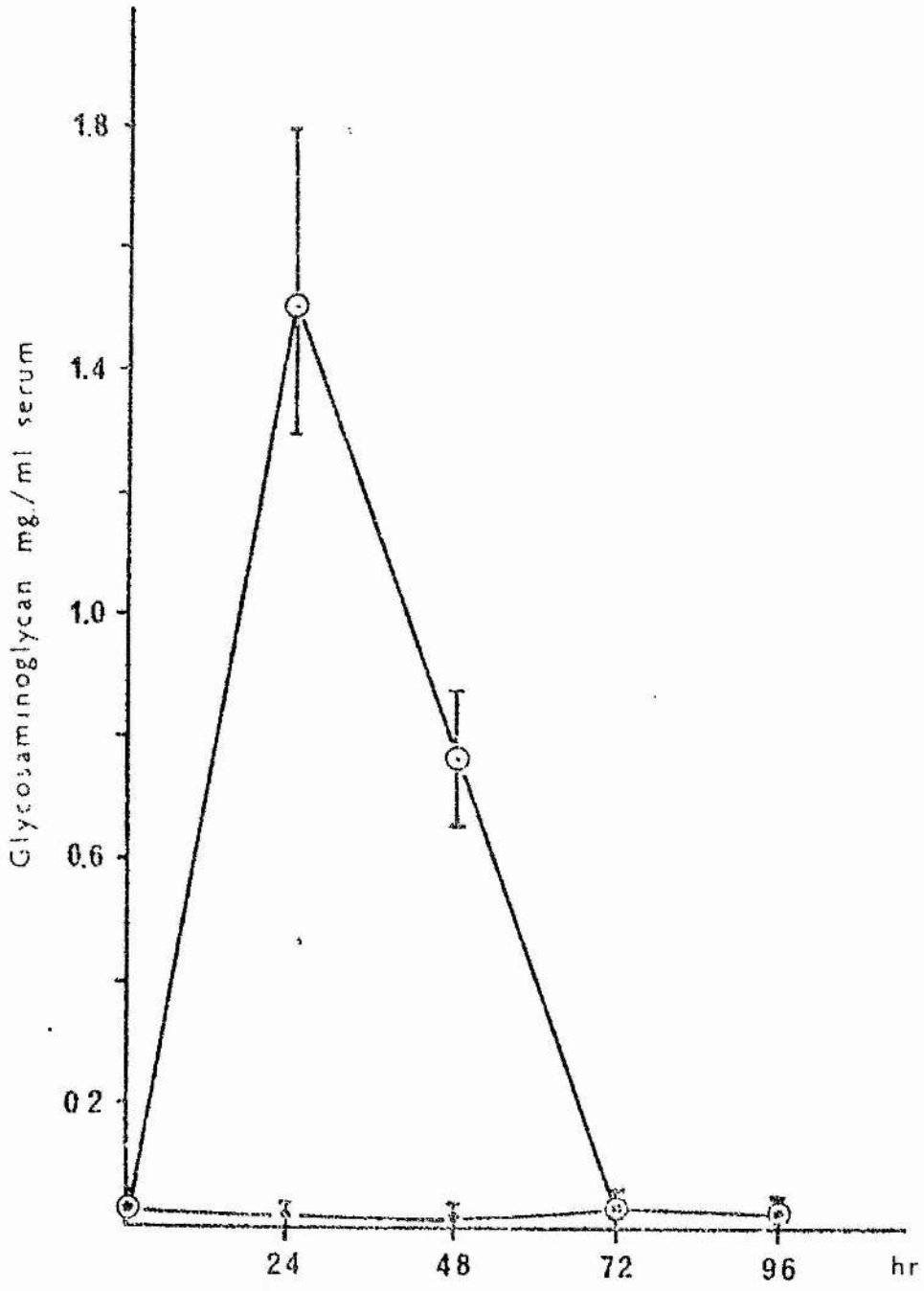
Fractions collected after gel filtration of the proteoglycan preparations isolated from radiosulphate-treated rabbits were emulsified in scintillant and counted in a Nuclear Chicago MK1 scintillation counter at 4^{o}. Aliquots of 0.5 ml. were removed from each fraction and mixed with 5 ml. of the counting mixture which consisted of toluene—Triton-X—100—ethanol (8:4:3, v/v/v) and contained 0.4% (w/v) PPO and 0.01% (w/v) POPOP (Patterson & Greene, 1965).

RESULTS

The release of chondroitin sulphate into the blood stream of the papain-treated rabbits was quantitatively monitored over a period of 96 hr. The glycosaminoglycan blood levels observed in the present study (Fig. 13) were basically identical to those previously reported, under similar experimental conditions, by Bryant, Leder & Stetten, (1958). Normal levels were restored after approx. 72 hr. It has been demonstrated that maximum depletion of cartilage matrix occurs 12 hr. after papain injection and that the cellular biosynthetic activity normally starts compensating for losses after further 12 hr. (Tsaltas, 1958). To avoid contamination of newly synthesized proteoglycan with enzymic degradation products still present in the tissue, papain-treated rabbits were kept under cortisone treatment for five days.

The yields of the preparations extracted from control and papain-treated cartilages, by homogenization with either water or cetylpyridinium chloride, are reported in Table 6. The results of chemical analyses on selected preparations are summarized in Table 7. The water-extracted preparations were further analyzed by infrared spectroscopy and prominent bands were observed at 725, 852 and 930 $cm.^{-1}$

Figure 13



Serum glycosaminoglycans in rabbits.

· Controls.

⊙ Papain-injected animals.

Table 6. Extraction yields

Values are expressed as mg./g. of dry cartilage.

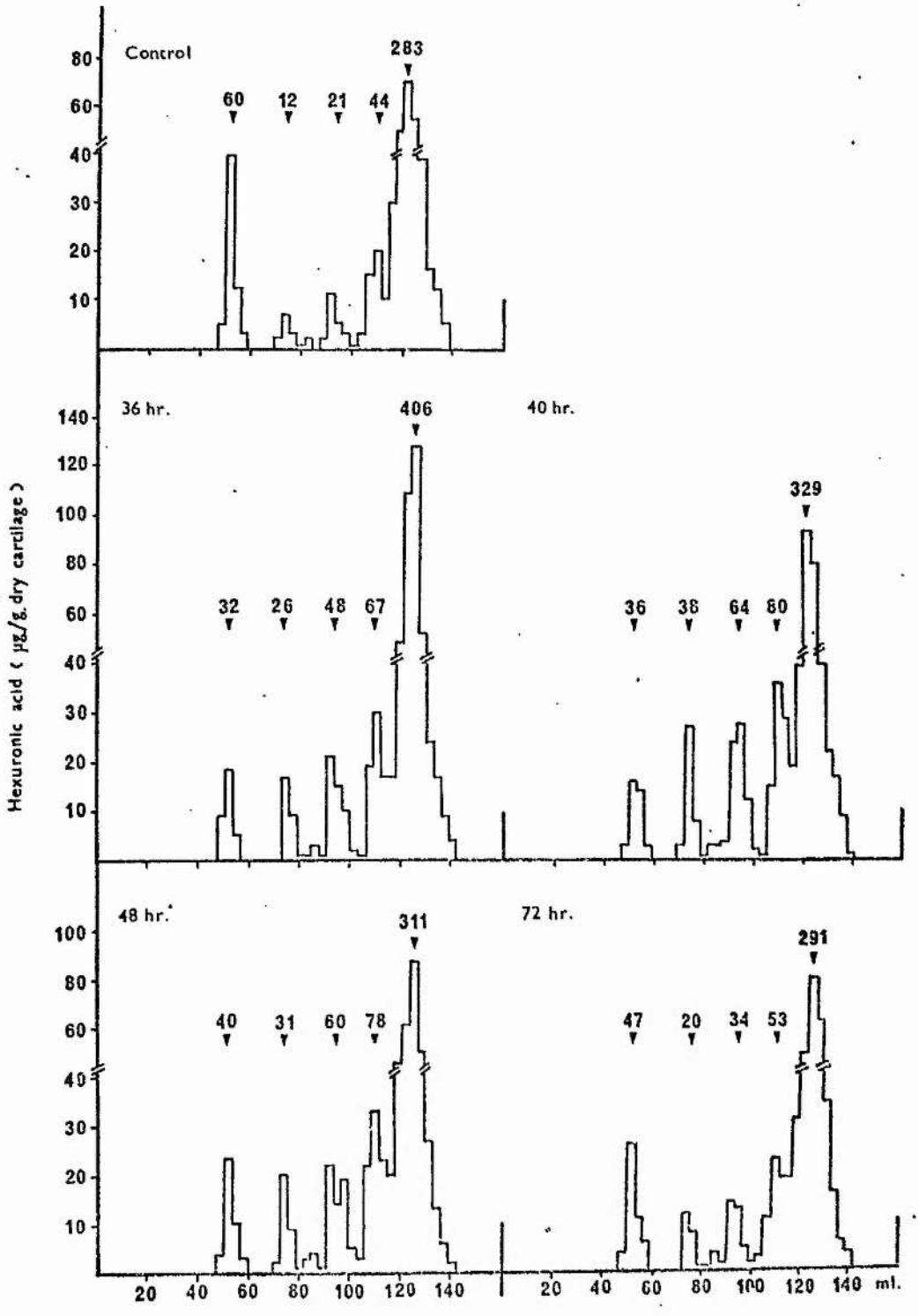
Sample	water extracted material	cetylpyridinium extracted material
Time elapsed after cortisone injection		
Control	5.15	36.62
36 hr.	8.78	26.95
40 hr.	8.50	27.42
48 hr.	6.60	31.37
72 hr.	5.83	35.80

Table 7. Chemical analyses

Values are expressed as percentages of ash and moisture-free samples.

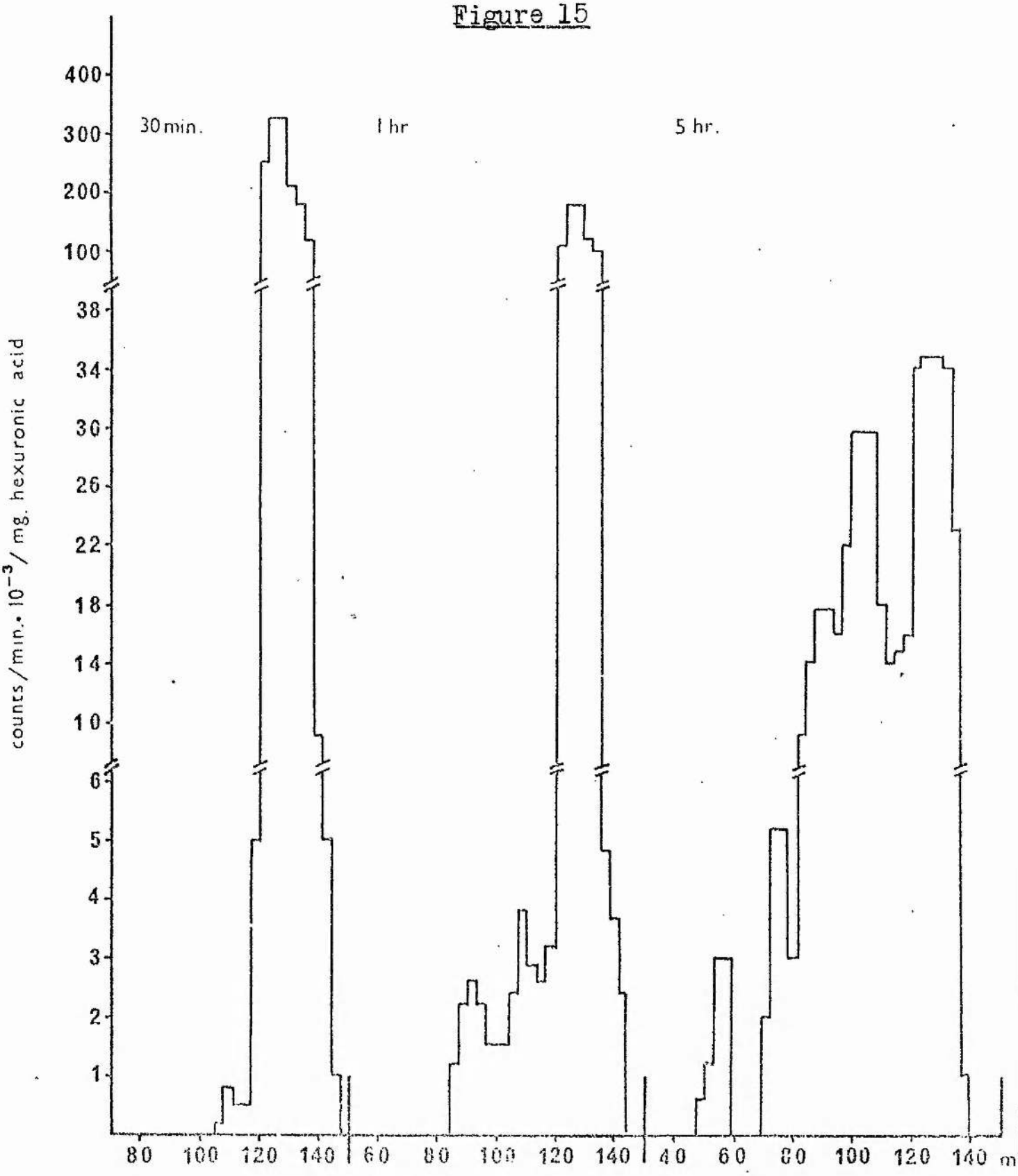
Sample	water extracted material		cetylpyridinium extracted material	
	Control	36 hr.	Control	36 hr.
Protein	40.01	43.78	17.25	17.30
Hexosamine (as free base)	8.55	6.84	24.11	24.06
Hexuronic acid	8.21	6.67	19.92	19.67
Hexose (as galactose)	31.29	31.64	6.20	6.17
Sulphur	0.96	0.77	4.30	4.26

Figure 14



Elution profiles of the water-extracted, hexuronic acid-containing materials from a 4% agarose column (bed volume 124 ml., void volume 52 ml.). Solvent: 1M potassium acetate, pH 7. Values above arrows refer to the amount of hexuronic acid recovered under each peak.

Figure 15



Specific activities of the hexuronic acid-containing materials extracted with water from the ear cartilage of rabbits treated with papain and subsequently injected with $Na_2{}^{35}SO_4$. The experimental chromatographic conditions are identical to those reported in the legend to Fig. 14.

The elution profiles of the water-extracted hexuronic acid-containing material on 4% agarose are reported in Fig. 14. The time indicated in each section of the figure refers to the time elapsed between the last cortisone injection and the sacrifice of the animals. The area under each peak was integrated and the values obtained are reported above the corresponding fractions. These values represent the amount of each macromolecular species present in the tissue, on a dry-weight basis.

The cetylpyridinium-extracted proteoglycan behaved as an excluded fraction when chromatographed on both 4% and 1% agarose.

The results of the radiosulphate incorporation into the water-extracted material are reported in Fig. 15. The time indicated in each section now refers to the time elapsed between the injection of $^{35}SO_4^{2-}$ and the sacrifice of the animals.

The proteoglycan extracted by cetylpridinium chloride showed no significant radioactive label incorporation in the 30 min. and 1 hr. experiments. After 5 hr., a specific activity of 3 000 counts/min./mg. of hexuronic acid, similar to that of the excluded fraction of the water-extracted material, was found.

DISCUSSION

Following the work of Gross, Mathews & Dorfman (1960) on the biosynthesis of the chondroitin 4-sulphate-protein complex in cartilage, other reports have shown that the protein and the glycosaminoglycan moieties of this complex are synthesized simultaneously (Campo & Dziewiatkowski, 1962; Telser, Robinson & Dorfman, 1965; de la Haba & Holtzer, 1965). However, the question is still open on whether chondrocytes produce a continuous protein core, to which some 20 to 60 polysaccharide chains are covalently bound (Mathews & Lozaityte, 1958; Partridge, Davis & Adair, 1961; Meyer, 1966; Serafini-Fracassini & Smith, 1966; Luscombe & Phelps, 1967; Serafini-Fracassini, Peters & Floreani, 1967), or whether smaller glycosaminoglycan-peptide sub-units are independently synthesized and then polymerized at a later stage.

To gain an understanding of this aspect of the cartilage biosynthetic mechanism, it was necessary to analyze the molecular weight distribution pattern of the complexes present in the tissue, within the molecular weight range in which sub-units were expected to be found (see Part 1).

Estimation of the number of components and their relative amounts in such an heterogeneous system was achieved by a study of the elution profiles of cartilage extracts on a calibrated agarose column, as previously described in Part 1.

The cartilage examined in the present investigation contained 3% hexosamine, on a dry-weight basis, this value being in keeping with that recently reported by Bentley & Rokosová (1970), which would imply a glycosaminoglycan content of approx. 84 mg./g. of dry cartilage. Only approx. 1.4% of this material was extracted by low-speed homogenization in water, while the yield was increased to approx. 30% by further extraction with cetylpyridinium chloride. Equally low yields have been previously reported (Tsaltas, 1958).

The composition of the water-extracted material showed hexosamine, hexuronic acid and sulphur contents compatible with the presence of chondroitin sulphate, contaminated by protein and other carbohydrate(s). Prominent bands at 725, 852 and 930 $cm.^{-1}$ in the infrared spectrum indicated that the isomer was chondroitin 4-sulphate (Mathews, 1958; Hoffman, Linker & Meyer, 1958). although chondroitin 6-sulphate is also known to be present

in the tissue (Bryant et al., 1958; Bentley & Rokosová, 1970). About 86% of this hexuronic acid containing material was retarded on 4% agarose revealing the presence of four low-molecular weight fractions having elution volumes of 72 ml. (fraction 1), 92 ml. (fraction 2), 109 ml. (fraction 3) and 125 ml. (fraction 4). In Part 1 a relationship was shown between the elution volumes, hydrodynamic volumes and molecular weights of proteoglycans of similar compositions and since fractions 1 and 3 co-chromatographed with the two preparations previously investigated, molecular weights of approx. 122 000 and 47 000 were respectively assigned. The molecular weight of fraction 2 was estimated to be approx. 82 000 by interpolation. As previously discussed (see Part 1), the macromolecules exhibiting a molecular weight of 47 000 are probably constituted by two glycosaminoglycan chains linked to a short protein core and since they embody the basic structural features of the proteoglycan macromolecule, they have been considered to be monomeric sub-units. On these grounds, the other two macromolecular species, revealed by gel filtration, could be interpreted as dimeric and trimeric sub-units. It is worth noticing that while

the dimer was never detected after treatment of cartilage proteoglycan with acid acetone (Serafini-Fracassini, 1968; Part 1), it appeared to be present in quite appreciable amount in the intact tissue.

The proteoglycan extracted with cetylpyridinium chloride was almost completely excluded both on 4% and 1% agarose indicating that the low-molecular weight fractions were completely removed by homogenization in water. The quantitative values reported for these fractions therefore represent true percentages of the cartilage dry weight.

It has been reported that depletion of the cartilage matrix by papain action enhances chondrocyte biosynthetic activity towards glycosaminoglycan production both *in vivo* and *in vitro* (McElligott & Potter, 1960; McElligott, 1962; Fitton Jackson, 1967; Guri & Bernstein, 1967; Bosmann, 1968). As clearly demonstrated by the elution profiles of the proteoglycan extracted from papain-treated animals, monomer, dimer and trimer were all increased in concentration when compared with controls. This rise in sub-units levels took place as soon as the cortisone treatment was discontinued, and reached a maximim after 40 hr. At 72 hr., values approaching those typical of controls were detected.

Meanwhile, the excluded fraction, representing a highly polymeric proteoglycan, increased steadily from a starting value of approx. 50% of the control level.

These changes in the tissue concentrations of the excluded material and of the low-molecular weight fractions, identical in size to those found in the control cartilage extract, make it unlikely that the latter represent merely enzymic degradation products. However, further and more conclusive evidence is furnished by the $^{35}SO_4^{2-}$ incorporation experiments conducted with papain-treated rabbits 40 hr. after the last cortisone injection, when sub-unit levels were at a maximum. The fact that no radioactivity was detectable in the cetylpyridinium-extracted material, representing 30% of the tissue proteoglycan, during the 30 min. and 1hr. experiments, casts doubt upon the possibility that the changes in radioactivity between the four retarded fractions could have arisen from breakdown of large protein-polysaccharide macromolecules. Furthermore, sulphate incorporation started from the low end of the molecular weight distribution profile and radioactivity was slowly transferred to monomer, dimer and trimer in a step-wise manner. In the 5 hr. experiment,

label was detected in the excluded fraction of the water-extracted material and in the proteoglycan extracted by cetylpyridinium chloride. If degradation did in fact occur in the tissue, the opposite pattern of label incorporation would have been obtained.

At present, characterization of fraction 4, which showed a very rapid rate of radiosulphate incorporation, has not been achieved, this being mainly due to contamination of the hexosamine-containing material with protein and anthrone-reacting compounds. However, its low molecular weight indicates that it probably consists of chondroitin sulphate biosynthetic intermediates.

These observations suggest that the macromolecular species referred to as monomer, dimer and trimer represent proteoglycan sub-units normally present in the tissue at a very low concentration. From the information which is at present available, their implication in the biosynthesis of the high-molecular weight cartilage protein-polysaccharide is speculative since no direct evidence of trimer polymerization into larger macromolecules has been obtained, apart from the detection of radiosulphate in the excluded

fraction at the same time of its incorporation into the trimer but with lower specific activity.

If polymerization of trimeric sub-units does take place, it remains to be seen whether it is occurring intracellularly or in the extracellular matrix. Since it has been demonstrated that newly esterified chondroitin sulphate is secreted from the chondrocytes about 30 min. after radiosulphate administration in vivo (Godman & Lane, 1964), it cannot be excluded that either the trimer or its precursors are the final products of cellular biosynthesis.

SUMMARY

1. Proteoglycans extracted from rabbit ear cartilage were analyzed by gel filtration. A highly polymeric material was identified together with four retarded fractions, three of which were found to have molecular weights of 122 000, 82 000 and 47 000 respectively. These were considered to represent trimeric, dimeric and monomeric sub-units.

2. After depletion of the glycosaminoglycans in the cartilage matrix, by papain treatment, and cortisone suppression of cellular biosynthetic activity for five days, the tissue concentrations of monomer, dimer and trimer were found to have risen well above control levels and to have reached a maximum 40 hr. after the last cortisone injection. They then slowly reverted to normal values. On the other hand, the amount of extracted polymeric proteoglycan increased throughout the time interval investigated.

3. $^{35}SO_4^{2-}$ was injected into papain-treated rabbits when sub-unit levels were at their maximum. Label was transferred from monomer to polymer through the other two sub-units.

4. It was concluded that the sub-units of fractions 1, 2 and 3 could represent true biosynthetic intermediates and not enzymic degradation products.

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